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## Physiological Effects on the Expression of Aquaporin 1-Like HC-1 in Cope's Gray Tree Frog, *Hyla Chrysoscelis*

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**PHYSIOLOGICAL EFFECTS ON THE EXPRESSION OF AQUAPORIN 1-LIKE  
HC-1 IN COPE'S GRAY TREE FROG, *HYLA CHRYSOSCELIS***

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science

By  
**RAM NARESH PANDEY**  
M.S. Eastern New Mexico University, 2001

2009

Wright State University

SCHOOL OF GRADUATE STUDIES

April 13, 2009

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY  
SUPERVISION BY RAM NARESH PANDEY ENTITLED **PHYSIOLOGICAL**  
**EFFECTS ON THE EXPRESSION OF AQUAPORIN 1-LIKE HC-1 IN**  
**COPE'S GRAY TREE FROG, *HYLA CHRYSOSCELIS*** BE ACCEPTED IN  
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF  
Master of Science.

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## ABSTRACT

Pandey, Ram Naresh, M.S. Department of Biological Sciences, Wright State University, 2009.  
Physiological Effects on the Expression of Aquaporin 1-like HC-1 in Cope's Gray Frog, *Hyla chrysoscelis*

Aquaporin 1 is a cell membrane integral protein, which functions for water transport through the hydrophobic cell membrane to the cytoplasm. HC-1 is an aquaporin 1-like protein expressed in *Hyla chrysoscelis* and amphibian that survives at sub-freezing temperatures in its natural habitat. The goal of the current study was to investigate the expression of HC-1 during changing physiological conditions. The expression level of HC-1 was determined in skin, muscle, liver, kidney, and intestines of warm, cold, frozen, and dehydrated frogs using Western blot and immunohistofluorescence. Although HC-1 was widely distributed, the expression level varied among tissues and physiological conditions. In addition, HC-1 was present in both glycosylated and deglycosylated forms, the pattern of glycosylation also varied with tissues and physiological conditions. Thus, I conclude that HC-1 is widely expressed in tissues of *Hyla chrysoscelis*, and that it is regulated in these tissues according to physiological demands.

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## INTRODUCTION

### **Cope's Gray Tree Frog**

*Hyla chrysoscelis* is a tree frog (order Anura, family hylidae) found in the Eastern United States. The range of distribution of *H. chrysoscelis* extends across a variety of habitat types, from subtropical southeastern USA to southern Canada (fig. 1), and including the lower elevation Piedmont and Coastal Plain of Virginia and the Carolinas. These tree frogs have woodland habitats, though they sometimes travel to more open areas to reach a breeding pond. The frogs are slightly sexually dimorphic. Males have black or gray throats in the breeding season, while the throat of the female is lighter (Tynning 1990). It is generally believed that *H. versicolor*, a tetraploid relative with similar physiological characteristics (Gerhardt et al. 1994, Harding 1997), evolved from *H. chrysoscelis* sometime during the last major ice age, when areas of extremely low temperature divided populations.

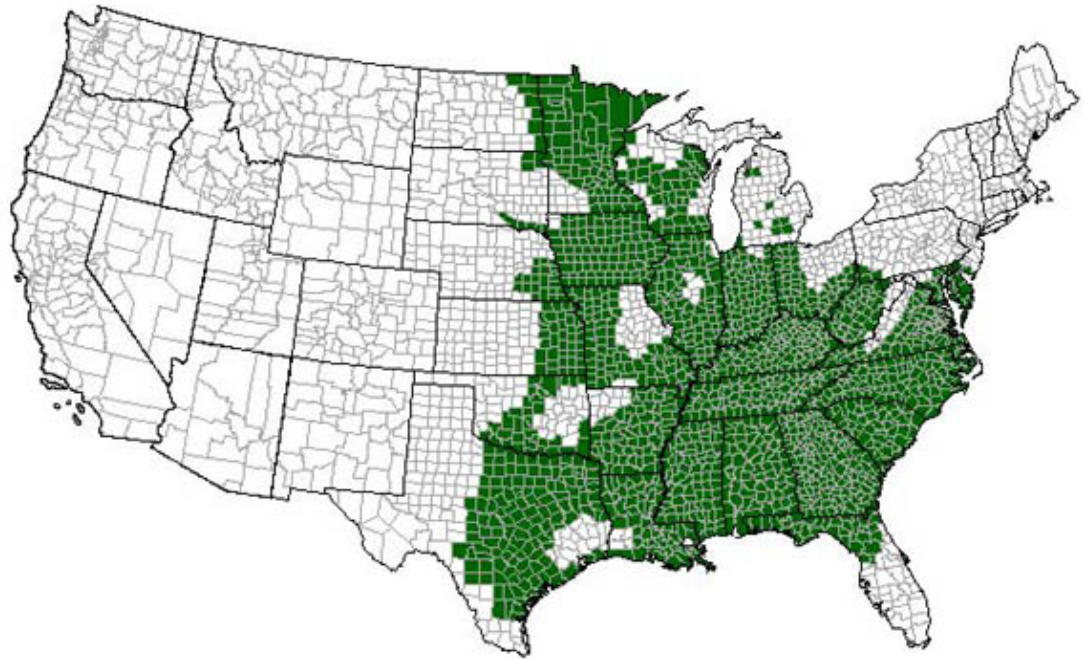


Figure 1. Distribution of *Hyla chrysoscelis* in USA. Green regions represent the habitat of *H. chrysoscelis*. (Source: [www.pwrc.usgs.gov/armiatlas/maps](http://www.pwrc.usgs.gov/armiatlas/maps))

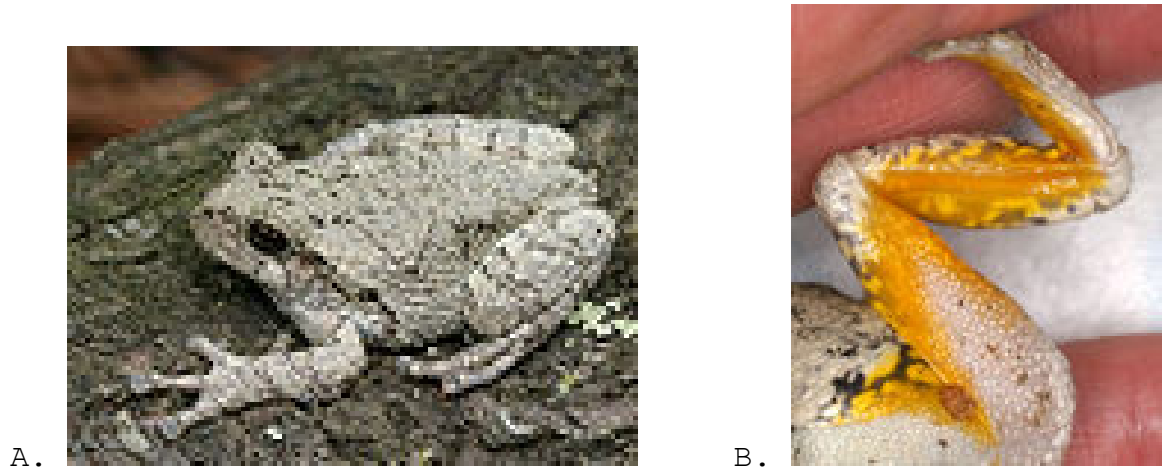


Figure 2. A. *Hyla chrysoscelis*. B. Hind limb of *H. chrysoscelis* showing orange color, one of the species' defining characteristics (Martof et al.1980).

*H. chrysoscelis* is reported to be slightly smaller, more arboreal, and more tolerant of dry conditions than *H. versicolor* (Martof 1980). The two forms of gray tree frog do not interbreed and are recognized as different species.

*H. chrysoscelis* is able to survive freezing (Gerhardt et al. 1994; Harding 1997). *H. chrysoscelis* might have adapted to freezing environments as it survived in the ice age (Gerhardt et al. 1994, Harding 1997). Mechanisms of freeze tolerance may have evolved from more general amphibian responses to dehydration (Churchill and Storey 1993)

Glucose and glycerol were measured in blood plasma of *Hyla chrysoscelis* collected from Minnesota, Indiana and Ohio (Irwin and Lee 2003, Zimmerman et al. 2007) and in *H. regilla*, another apparently freeze-tolerant hyliid (Croes and Thomas, 2000) from Northern California. The two species showed two different patterns of glycerol accumulation in blood plasma. The concentration of glucose increased upon freezing in the both species (Croes and Thomas 2000, Irwin and Lee 2003, Zimmerman et al. 2007). In contrast, in *H. chrysoscelis* and in *H. versicolor*, glycerol accumulated during cold acclimation prior to freezing (Zimmerman et al. 2007, Irwin and Lee 2003); thus, cold-acclimated *H. chrysoscelis* had plasma glycerol levels of about 50 mmol/L,

whereas plasma glycerol level was below 1 mmol/L in *H. regilla* (Croes and Thomas 2000, Zimmerman et al. 2007).

In *H. versicolor*, freezing induced a rise in liver glucose and glycerol production in winter. The increase in glucose and glycerol was accompanied by a significant decline in liver glycogen of *H. regilla* (Croes and Thomas 2000). These findings suggest that the liver is the organ responsible for cryoprotectant synthesis. The rise in plasma glucose, along with increased levels of liver glucose and glycerol in response to freezing, suggests that these compounds are being used as cryoprotectants (review Krane and Goldstein 2007, Zimmerman et al. 2007). The compounds accomplish that function in part by stabilizing cell membranes and intracellular structures. However, another important function is to help in the regulation water exchange between the intracellular and extracellular compartments. Extracellular accumulation of cryoprotectants increases osmotic concentration, which helps to promote movement of water to the extracellular space during freezing. At that time, water must move out of the cells quickly to prevent intracellular ice formation. To meet this high demand for flow of water, cells need mechanisms in the cell membrane for water to cross the hydrophobic barrier. In this situation water

channel proteins are the major source of high exchange of water.

Thus, to preserve physiological integrity during cold acclimation and freezing, *H. chrysoscelis* must transport glycerol and water. In the present study, I examine the expression of one protein involved in the transport of water, and evaluate whether expression of that protein is regulated according to physiological condition.

### **Aquaporin 1**

Water molecules are required for cell metabolism but cell membranes are hydrophobic in nature. Nevertheless, although water moves across the tight junction through the paracellular pathway (Guo et al. 2003, Orce et al. 2004), the major exchange of water occurs transcellularly. Biophysical, physiological, and electron microscopic measurements reveal that water movement is mediated by membrane transport channels. These channels were originally identified as "aggregates" (Chevalier et al. 1974, Brown et al. 1983, Yasui 2004), but more recently the aggregates have been discovered as aquaporins (Agre et al. 1993), membrane integral proteins that cross the hydrophobic barrier of the plasma membrane (Agre and Kozono 2003, Krane and Kishore 2003). Aquaporins are ubiquitously distributed

from microorganisms to plants and animals. Vertebrate aquaporins can be divided into two types: orthodox aquaporins that create pores with high specificity for water and aquaglyceroporins that form pores to allow entry of glycerol and water through the cell membrane.

Aquaporin 1 is one of the orthodox proteins, responsible for water transport (fig. 3). Aquaporin 1 was first investigated and purified in human red blood cells and renal proximal tubules (Dneker *et al.* 1988). Aquaporin 1 is a tetramer protein and each subunit makes a water channel. These monomers are arranged side by side in a tight cluster, with the pores running parallel. Each monomer comprises six membrane-spanning helices that partially surround two shorter helices (fig 4). The short non-membrane-spanning helices make up the major portion of the pore. Each pore has a dumbbell-like shape. One broad end is the cytoplasmic vestibule; the other is the extracellular vestibule. The central pore formed by the tetramer may function as an ion channel (Anthony *et al.* 2000, Zhang *et al.* 2007).

A series of carbonyl oxygens form a hydrophilic path across the pore region and through the rest of the selectivity filter. One of the oxygens, along with a



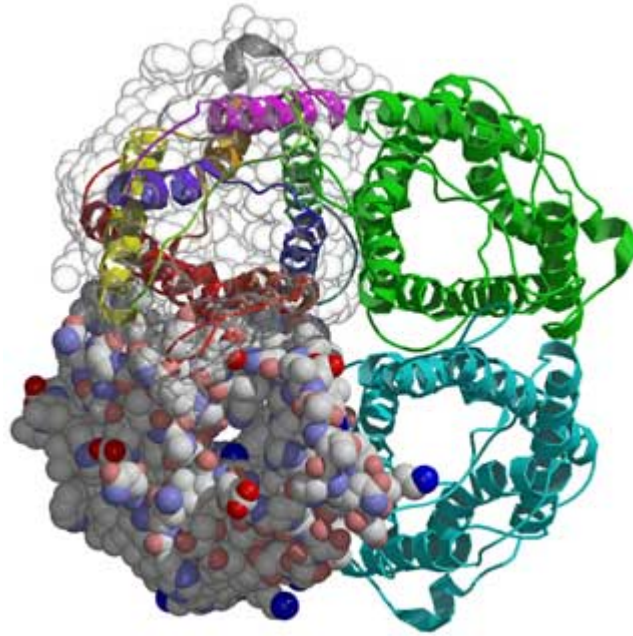


Figure 3. The AQP1 tetramer. A view looking down the pores from the cytoplasmic side to the membrane. One monomer of the four is represented as a solid space-filling model (Sui *et al.* 2001).

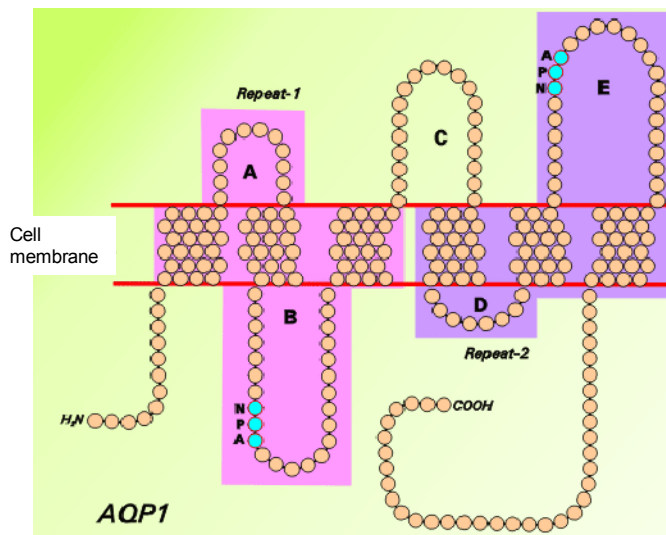


Figure 4. Six membrane helices, extracellular, and cytoplasmic location of aquaporin 1. The 2003 Nobel Prize in Chemistry was awarded to Peter Agre for the discovery of aquaporins and jointly to Roderick MacKinnon for his work on the structure and operation of ion channels.

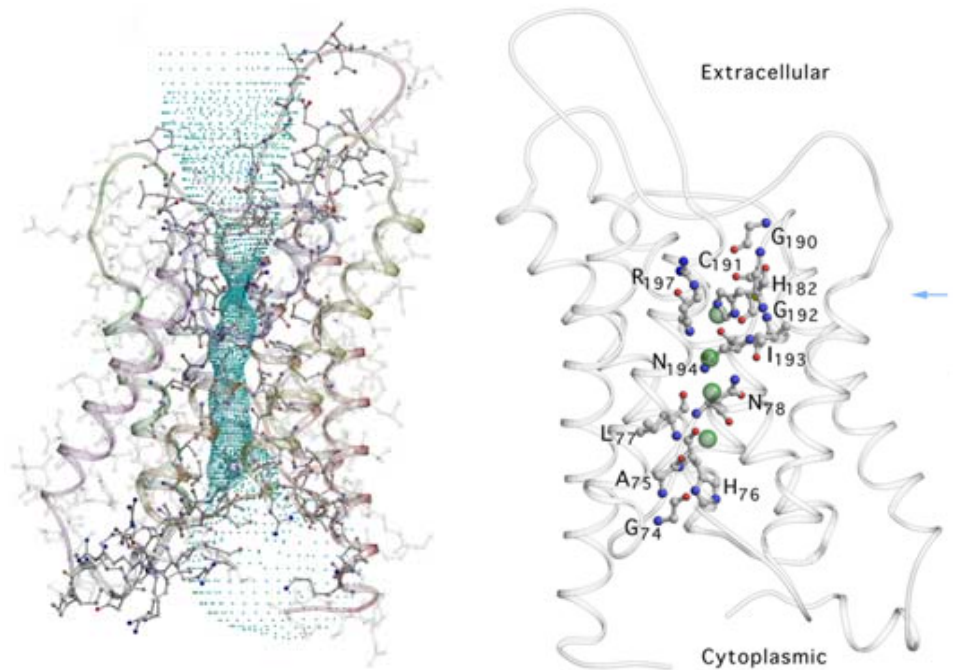


Figure 5.A. Side view of aquaporin 1 showing the pore (turquoise dots) and the residues that line the pore (opaque ball-and-stick structures). The extracellular vestibule is above and the cytoplasmic is below. The pinched area with the highest concentration of turquoise dots is the constriction region. B. The hydrophilic path across the selectivity filter, highlighted by ball-and-stick structures of the side chains involved. The green spheres are the water molecules observed during transport through membrane. The constriction region is indicated by the blue arrow (Sui et al. 2001).

histidine residue and an arginine residue, forms the hydrophilic face of the constriction region. Opposite this face is a hydrophobic face formed by a phenylalanine residue. Three of the four residues that form the constriction region (the arginine, histidine, and phenylalanine residues) are conserved in all known water-specific aquaporins. This observation suggests that the presence of these residues can be used as a marker for identifying other water-specific aquaporins (fig. 5, Sui *et al.* 2001). The rate of water flow through the water channel is in the range of  $10^9$ - $10^{10}$  molecules per second per channel (Heymann and Engel 1999).

## **Rationale**

Aquaporin 1 is widely distributed in mammalian tissues such as renal proximal convoluted tubules and the descending thin limbs of the loop of Henle in rat (Dneker *et al.* 1988), dog (Higa *et al.* 2000), and human (Maunsbach *et al.* 1997), human mammary gland (Mohasheri and Marples, 2004), the mouse gallbladder (Calamita *et al.* 2005), the retina (Kim *et al.* 1998), iris in the eye (Yamaguchi *et al.* 2006), and choroid plexus (Speake *et al.* 2003) in the brain

of rats, and in the brush border, and basolateral membranes of non-ciliated cells in the rat efferent duct (Brown *et al.* 1993).

Amphibians are the first animals that adapted to the terrestrial environment from the aquatic environment, and some amphibians even survive in severe cold and dry conditions in the terrestrial ecosystem. It is thus a matter of interest to investigate the water transport system for their adaptation. In amphibians, an AQP1-like protein has been localized in pelvic skin and urinary bladder of the tree frog *Hyla japonica* (Hasegawa *et al.* 2003) and *H. chrysoscelis* (Zimmerman *et al.* 2007). Those proteins were characterized by expressing them in *Xenopus* oocytes (Hasegawa *et al.* 2003, Zimmerman *et al.* 2007), which confirmed their function as classical water channels, with high transport of water relative to glycerol.

In *Hyla chrysoscelis*, my study species, the aquaporin identified by Zimmerman *et al.* (2007) was designated as HC-1. It has high homology to mammalian aquaporin 1 and 98% amino acid similarity to the water channel aquaporin 1 of *H. japonica* (Tanii *et al.* 2002). HC-1 mRNA was, at first, cloned from kidney of warm *Hyla chrysoscelis*, but subsequent PCR expression studies indicated that it is

expressed widely among tissues of this animal (fig. 6, Zimmerman *et al.* 2007).

As noted above, *H. chrysoscelis* is a freeze tolerant tree frog. When the animal freezes, ice forms in the extracellular space. This raises the solute concentration in unfrozen solution extracellularly (Mazur 1984), and water then exits cells by osmosis. The loss of cell water helps to prevent ice formation inside cells, which would be disruptive to cell structure (Layne and Jones 2004). The ability to tolerate freezing may be related to more general amphibian mechanisms to respond to water loss by evaporation. Although expression of HC-1 mRNA has been reported in some tissues of *H. chrysoscelis*, expression of HC-1 protein in tissues of *H. chrysoscelis* has not been investigated. Recently, activation of ion channel function of mammalian aquaporin 1 by PKC has been reported in *Xenopus* oocytes (Zhang *et al.* 2007). This finding that function of mammalian aquaporin 1 (often considered a "housekeeping," constitutive protein) may be regulated, along with the freeze-induced demand for water transport in perhaps all organs of *H. chrysoscelis*, led us to hypothesize that aquaporin 1 -like HC-1 might be regulated in different physiological conditions in the gray tree

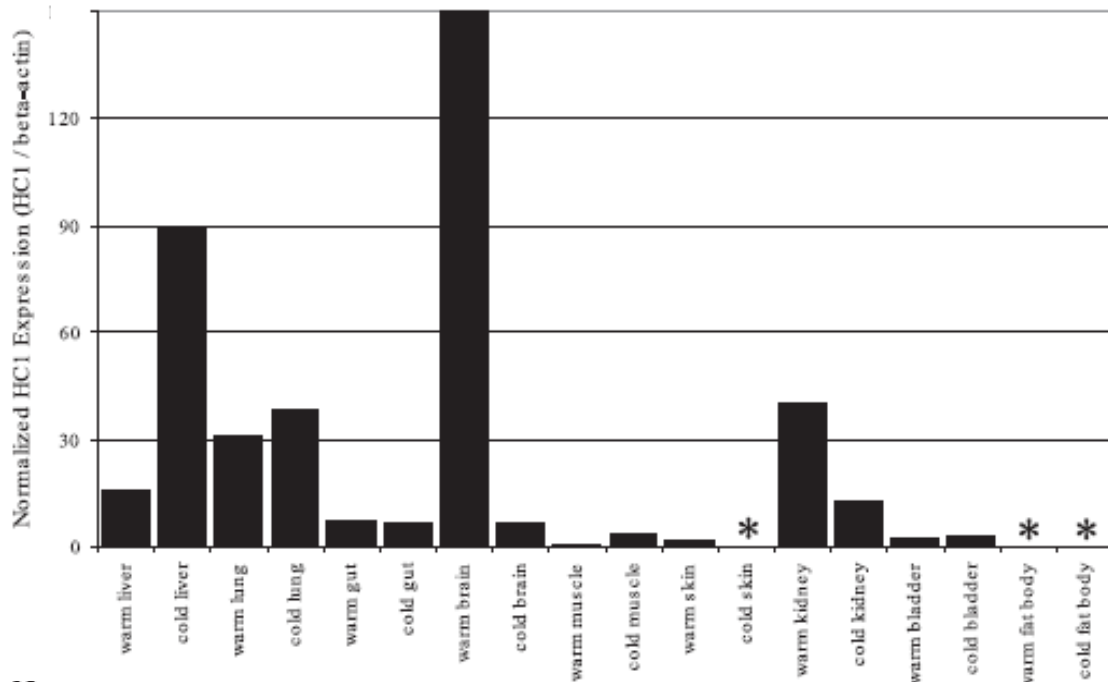


Figure 6. Expression of HC-1 mRNA in different tissues of warm and cold *H. chrysoscelis* (Zimmerman et al. 2007).

frog. Therefore, I hypothesized that aquaporin 1-like HC-1 would be expressed widely in tissues of *H. chrysoscelis* and its expression would be modulated by changing physiological circumstances like dehydration, cold acclimation, and freezing.

### **Aims**

The following aims were established to examine the hypothesis for the regulation of HC-1 by the physiological circumstances of *H. chrysoscelis*.

- 1) To investigate the distribution and immunolocalization of HC-1 protein in skin, kidney, liver, muscle, and intestines of *H. chrysoscelis*.
- 2) To compare the expression level of HC-1 among different physiological circumstances in different tissues.

## MATERIALS AND METHODS

### ***Animal***

Grey tree frogs, *Hyla chrysoscelis*, were collected from Greene County, Ohio and were kept in the laboratory using standard housing criteria, as approved by the Animal Care and Use Committee of Wright State University. A single or a pair of frogs was housed in a single cage having a dimension of 60 cm X 30 cm X 30 cm with a vented top. Frogs were exposed to a light cycle of 12 hr light/12 hr dark at 22°C. They had free access to water and were fed crickets twice per week. Frogs were inspected daily to document healthy conditions.

### ***Frog dehydration***

For slow dehydration, urine from the urinary bladder was removed by inserting a glass capillary tube into the cloacae (standard procedure for collecting amphibian urine; Walker 1940, Stiffler 1991), and the frogs were weighed to provide "standard mass." We then applied two dehydration protocols, designed to induce either rapid or slow dehydration (see below). Each dehydration protocol was



continued until the body mass of the frog dropped by 20% (Seebacher and Alford 2002). Dehydration was accomplished with fasting because the insect food would provide a rich source of water that would prevent dehydration.

To dehydrate frogs slowly, animals with bladders drained were allowed to regain urine for one hour, and then transferred to a cage with a vented top without water or food. Each day the frogs were weighed, being careful not to expel urine, until the mass of the frog declined to 80% of standard mass. This dehydration process was completed in approximately seven days.

For fast dehydration, water from the bladder was removed and whole animal was weighed as described above. The frog was then placed in a plastic container with a vented top and a bed of desiccant, calcium sulphate ( $\text{CaSO}_4$ ), with a wire mesh approximately 3 cm above the desiccant restricting the direct contact between the frogs and desiccant. The animal was weighed until the body mass of each frog reduced to 80% of standard mass. The fast dehydration process was accomplished within 2 days.

### **Cold acclimation**

To acclimate animals to the cold, we moved them into a temperature-controlled room and gradually shifted from room

temperature ( $\sim 20^{\circ}\text{C}$ ) to  $4^{\circ}\text{C}$ . The temperature shift was accomplished over a period of approximately eight weeks by dropping the temperature at two weeks intervals; light cycle was also changed over that time period to approximate a natural seasonal transition, which may be necessary to induce cold acclimation characterized by glycerol accumulation. As the temperature was decreased, frogs had access to water and food until the frogs ceased feeding ( $\sim 6\text{--}8^{\circ}\text{C}$ ). The temperature was decreased to  $4^{\circ}\text{C}$  and remained at  $4^{\circ}\text{C}$  until the frogs were sacrificed or conditioned for freezing.

Freezing was induced by reducing the temperature by one degree Celsius per day until it reached  $-2.5^{\circ}\text{C}$ . At that point, a piece of ice was touched to the dorsum of the super cooled frog, and this triggered ice nucleation.

Six organs--skin, liver, small intestine, large intestine, kidney, and skeletal muscles (combined anterior and posterior muscles of the thigh)--were collected from frogs in each physiological condition, for analysis by histology, immunofluorescence, and Western blot to assess expression of aquaporin 1. These organs were selected to represent several classes of function. The skin, an important osmoregulatory organ, is directly exposed to the environment; environmental stress is exerted first on skin.

Kidneys have central roles in regulating body fluid homeostasis, including fluid exchange via blood filtration and tubular reabsorption of water and solutes. Liver produces cryoprotectants, and exchange of water with increasing concentration of cryoprotectants may be required during transport of cryoprotectants. Muscle does not have a specialized osmoregulatory role but may need to transport water and glycerol for cryoprotection. Finally, intestines absorb digestive liquids and therefore are expected to have a high exchange of water, especially associated with feeding.

### ***Reagents and antibody production***

A peptide consisting of the 16 c-terminal amino acids from HC-1 was synthesized, and antibody was generated against the synthetic peptide in rabbits (Sigma Genosys, Woodlands, TX). The HC-1 peptide sequence selected for antibody production was **CYELDGEDARMEMKPK**. Antiserum was purified using an immunoprecipitation technique against HC-1 peptide using SulfoLink Immobilization Kit (Pierce, Rockford, IL) and stored at -80°C. Goat and donkey serum, Cy2-conjugated goat anti-rabbit antibody, and Cy5-conjugated donkey anti-rabbit antibody were purchased from Jackson ImmunoResearch laboratories. Fluoromount-G was

obtained from SouthernBiotech for slide mounting. Propidium iodide and protease inhibitor cocktail (P8340) were obtained from Sigma. Prestained protein ladder was purchased from Fisher. SDS-PAGE material was purchased from BioRad and BCA protein assay kit was obtained from Pierce. Mouse monoclonal  $\beta$ -actin (ab8224) was purchased from Abcam and donkey anti-mouse-HRP antibody (SA1-1000) was obtained from Affinity BioReagents.  $\beta$ -integrin (sc-8978, Santa Cruz Biotech, CA) and  $\alpha$ -tubulin (sc-5546, Santa Cruz Biotech, CA), and donkey anti-rabbit-HRP antibody were bought from Santa Cruz Biotechnology.

### ***Protein analysis using Western blot***

Three frogs from each physiological condition were dissected and tissues from each frog were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Tissue was lysed with plasma membrane lysis buffer (25 mM Tris-HCl, pH 7.2, 125 mM NaCl, 10% glycerol, 1% Triton X-100, and 1 mM EDTA). Total protein was quantified with BCA (Pierce, Rockford, IL) and 30  $\mu\text{g}$  of protein was mixed with loading buffer (62 mM Tris-HCl, pH 6.8, 0.01% bromophenol blue, 10% glycerol, 100 mM 2- $\beta$ -mercaptoethanol, and 2% sodium-dodecyl-sulfate (SDS)) before loading onto a gel. The protein mixture was separated on a 12% SDS-polyacrylamide gel by

electrophoresis at 150V for 1.5h along with prestained protein ladder (EZ-Run Prestained Rec Protein Ladder, Thermo Fisher, Waltham, MA). Proteins were transferred to polyvinylidene difluoride (PVDF) (Immobilon-P Transfer Membrane, Millipore, Bedford, MA), blocked with 4% nonfat dry milk in phosphate buffered saline (PBST; 137 mM, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4mM KH<sub>2</sub>PO<sub>4</sub>, 0.05% Tween-20, pH 7.3), then incubated with 0.14 µg/µl of primary antibody HC-1 in 2% nonfat dry milk prepared in PBST. The membrane was then treated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) prepared in 2% milk in PBST. Protein bands on blots were detected using enhanced chemiluminescence (ECL, Pierce, Rockford, IL) and imaged on an LAS-4000 luminescent image analyzer (Fujifilm Life Science USA, Stamford, CT). Specificity of binding was tested by incubating primary antibody with a 200-fold molar excess of antigen peptide for 1 hr at room temperature prior to application to the membrane. To confirm the presence of cell membrane proteins in lysate, the PVDF membrane was probed with rabbit anti-β-integrin antibody(sc-8978, Santa Cruz Bio, CA), which revealed a strong signal in each blot (data not shown). Further, the loading quantity was normalized by incubating with 0.1 µg mouse antibody against β-actin (ab8224, abcam, MA) or α-

tubulin (Sigma, MO). We used 0.04 µg of donkey anti-mouse secondary antibody conjugated with HRP (SA1-100, Fisher, PA) to detect actin and tubulin. The HC-1, actin, and tubulin bands were digitized using Fuzi Multigauge software, which measures the pixels of a fixed area for protein band quantification. The value of HC-1 bands was divided with the value of actin or tubulin, which provided a loading control. The value of the ratio was used for statistical analysis.

### ***Deglycosylation***

Because our Western blots often contained what appeared to be antibody-specific signals at both the nominal aquaporin molecular weight and at higher molecular weights, and because glycosylation of aquaporins is well recognized as part of the mechanism by which they are localized to the membrane (Hendricks *et al.* 2003), we examined the extent to which *N*-linked glycosylation contributed to this pattern of results. Twenty micrograms protein was mixed with denaturing buffer (0.5% SDS and 40 mM DTT) and heated at 100°C for 5 min for denaturation of protein. Finally, the solution was incubated in reaction buffer (50 mM sodium phosphate buffer with 1% NP40, pH 7.5)) with 1000 units of Peptide: N-glycosidase F (New

England BioLabs, Ipswich, MA) for 1 hour at 37°C. The same quantity of protein from each tissue was treated without enzyme as above. Digested and undigested proteins were loaded on 12% SDS-polyacrylamide gel.

### ***Histology***

Organs were dissected from frogs and immediately transferred to periodate-lysine-paraformaldehyde (PLP) fixative containing 4% paraformaldehyde, 75 mM lysine, 37.5 mM sodium periodate, and 10 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.2) (McClean and Nakane, 1974) for overnight at 4°C. The fixed organs were washed with water, dehydrated, and embedded in paraffin (Humason 1979). Four micrometer sections of each organ were deparaffinized and stained with hematoxylin and eosin (Humason 1979) to visualize the cellular framework of the organs. The stained organ was photographed by a light microscope attached with a camera (Nikon Labopot-2, Japan). Tissues were labeled using *Xenopus* histology book (Wiechmann and Wirsig-Wiechmann 2003).

### ***Immunofluorescence***

Ten micrometer sections of each organ were mounted on gelatinized slides, deparaffinized and rehydrated gradually with descending series of ethanol after removing the

paraffin with xylene. The sections were washed with phosphate-buffered saline (0.02 M sodium monobasic phosphate, 0.08 M sodium dibasic phosphate, and 150 mM NaCl, pH 7.2) and treated with 0.2% Triton X-100 following the further incubation with 0.1% sodium borohydride (pH 8) and 1% glycine. The tissue was incubated with blocking solution (10% serum, 4% BSA, and 0.05% Tween-20) for one hour at room temperature, followed by incubation with 0.08 µg of primary anti-HC-1 antibody overnight at 4°C. After washing, the organ section was stained with 0.06 µg of donkey anti-rabbit cy5-conjugated secondary antibody and propidium iodide for an hour at room temperature. The nuclei were stained with 0.1 µg of propidium iodide (Sigma-Aldrich, St. Louis, MO). The signal was visualized by a confocal microscope (TCS SP2, Leica, Wetzlar) at different magnifications. The specificity of HC-1 signal was determined by pre-absorbing 0.08 µg of the HC-1 antibody with 0.4 µg of HC-1 peptide for one hour at room temperature.

### ***Statistics***

One factor analysis of variance (ANOVA; Microsoft Excel) was applied to investigate the variation of HC-1 among warm, cold, frozen, slow dehydrated and fast



dehydrated frogs for ventral skin, liver, kidney, muscles, small intestine, and large intestine. Post-hoc comparisons were made using Newman-Keuls testing to analyze the variation among different treatments. In each case,  $\alpha$  value was fixed at 0.05 levels. The *p-value* <0.05 was considered as significant.

### ***Limitation***

Most animals were breeding males at the time of collection. They were detected from their calling tones at the breeding ponds. However, the health and age of individuals at the time of collection, which might influence cell physiology, was not known. Another point worth noting is that food was not withheld from warm-acclimated animals, while whereas dehydrated frogs did not eat, and so the warm frogs are not true controls.

## RESULTS

### ***HC-1 expression in warm frogs: Western blots***

HC-1 protein was expressed in a pattern of 28 kDa to 45+ kDa in the all examined tissues in warm frogs and these bands were blocked by HC-1 peptide (fig.7A). To test whether the higher molecular weight bands represent glycosylated protein, as previously described for several aquaporins (Hendriks *et al.* 2004, Lu *et al.* 2008, a review Takata *et al.* 2008), protein extracts from each organ were treated with N-glycosidase, an enzyme which cleaves the N-linked glycan molecule from a protein, prior to running a gel. The result was that the higher molecular weight bands largely disappeared after treatment with N-linked glycosidase, but the lower bands, representing deglycosilated aquaporin 1-like HC-1, appeared with stronger intensity (fig.7B).

### ***HC-1 expression at various physiological conditions:***

A similar result was found when an individual organ, such as kidney (fig. 8A), was examined under different

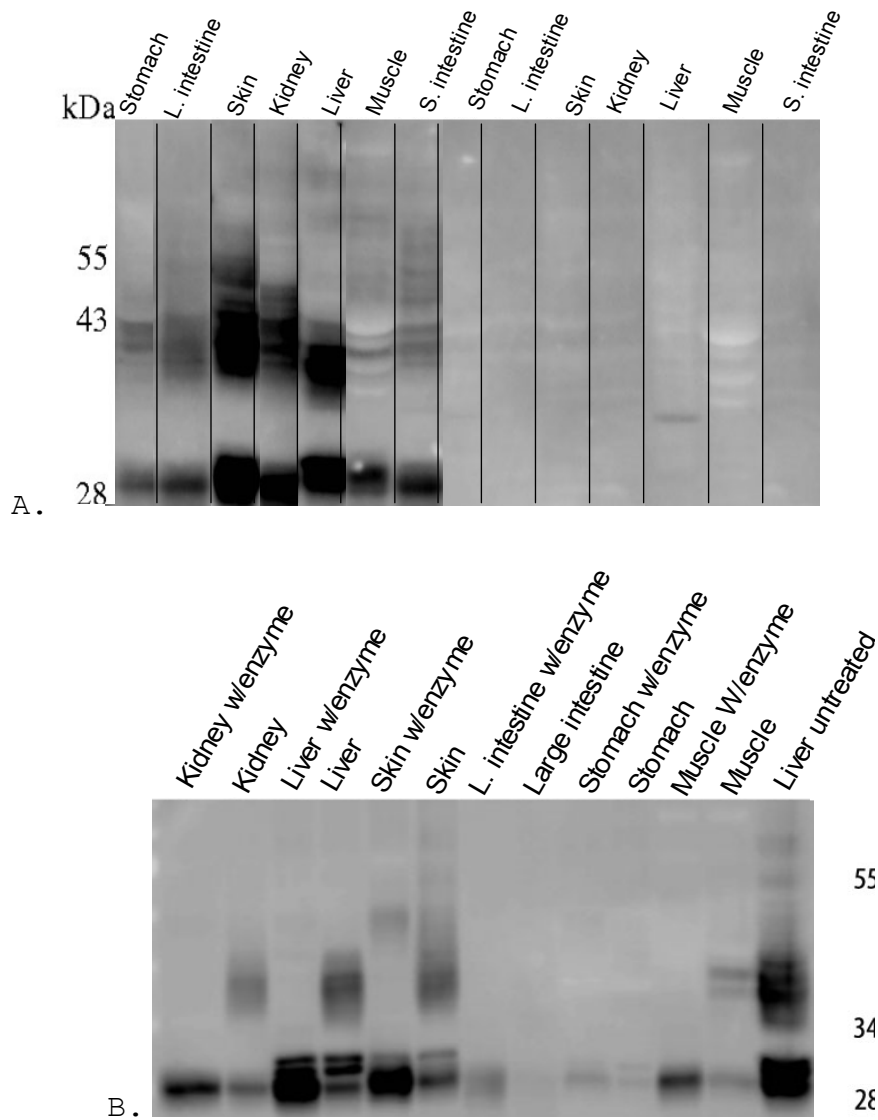


Figure 7. HC-1 expression and deglycosylation in warm frogs. A. HC-1 without (left) and with (right) peptide of HC-1 in warm frogs, B. Deglycosylated and glycosylated HC-1 in various tissues probed with HC-1. n = 3.

physiological conditions. The 28 kDa band was revealed in kidney from all physiological conditions, and the remaining protein bands were found in between 30 kDa and 55 kDa (fig. 8A). Those bands were blocked with the HC-1 peptide preincubated with HC-1 rabbit antibody confirming the specificity of the HC-1 signal on the Western blots (fig. 8A). All protein bands were digitized and the observed value was normalized relative to endogenous expression of actin to compare the regulation of HC-1 expression in various organs under different physiological circumstances.

Significant variation in HC-1 expression was observed among warm, cold, frozen, slow dehydrated and fast dehydrated conditions for kidney. In particular, warm, cold, and frozen animals had greater expression of HC-1 ( $p < 0.05$ ) compared with kidneys from slow and fast dehydrated animals (fig. 8B).

Similar to the kidney, the protein bands of HC-1 extended from 28 kDa to 45+ kDa in the ventral skin examined at various physiological conditions. An additional protein band of 30 kDa was found, which was absent in the kidney (fig. 9A). The expression of protein varied ( $p$  - value  $< 0.01$ ) among warm, cold, frozen, slow dehydrated,

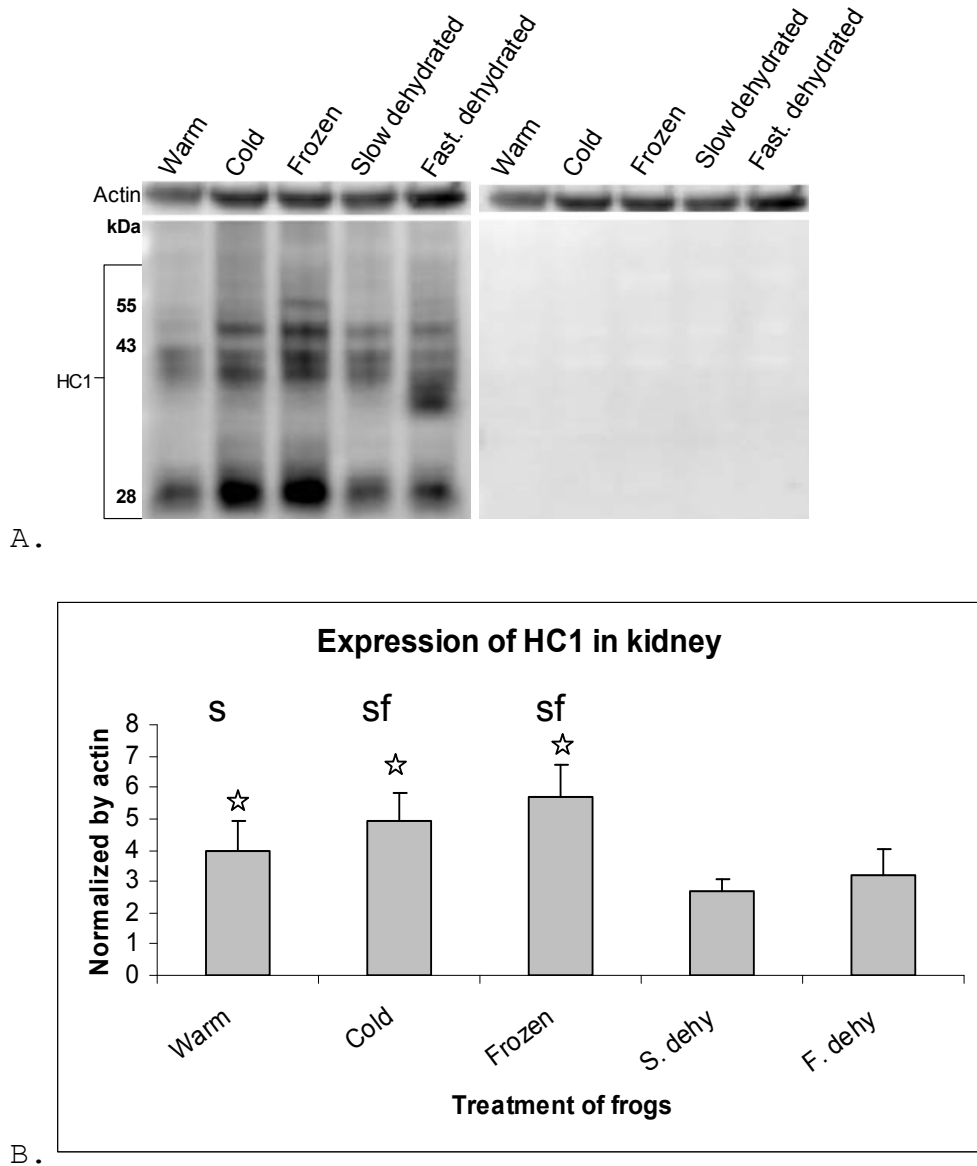
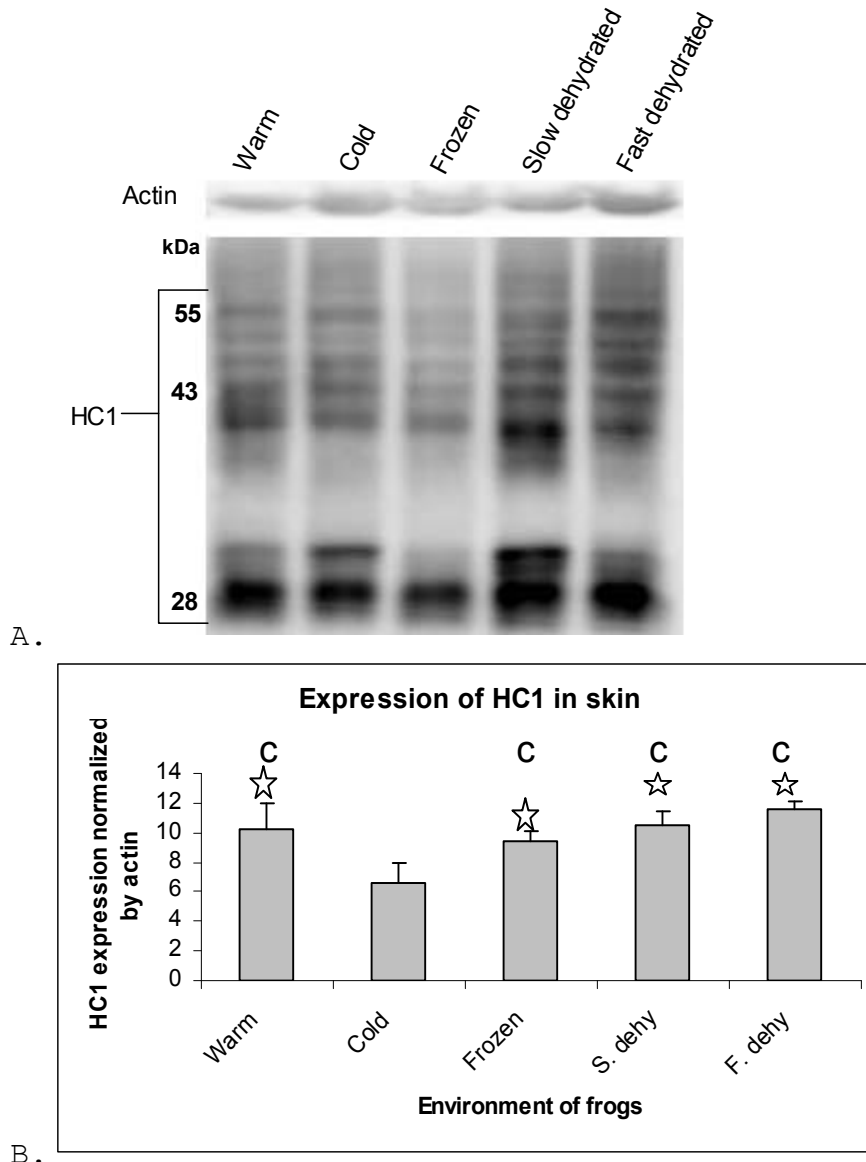


Figure 8. HC-1 expression in kidney. A. Thirty micrograms of protein were loaded in each well of 12% SDS-PAGE for each condition as indicated in the diagram. The right side of the membrane was incubated with the preincubated HC-1 with its peptide. The glycosylated bands are clustered in between 35 kDa to 45 kDa. B. Expression of HC-1 expression was quantified in reference to actin of frog ( $n = 3$ ). The  $p$ -value is 0.007 for ANOVA test at  $F_{4,10}$ . The  $p$ -value for warm vs slow dehydrated is  $<0.05$ . Cold and frozen in compare to slow dehydrated and fast dehydrated  $p$ -value is  $<0.01$ . s and f are slow and fast dehydrated respectively.



B.

Figure 9. HC-1 expression in ventral skin. A. Thirty micrograms of protein were loaded in each well of 12% SDS-PAGE for each condition as indicated in the diagram. The doublet band of HC-1 protein at the lower part of the membrane is common in all the cases except frozen tissue. The nascent protein 28 kDa is migrating to 31 kDa band in cold tissue. B. Expression of HC-1 expression was quantified in reference to actin expression in the tissues ( $n = 3$ ). The  $p$ -value generated by ANOVA ( $df: F_{4,10}$ ) test was 0.002. The  $p$ -value of cold (c) frog in comparison to the rest of conditions is  $<0.05$  by post hoc test.

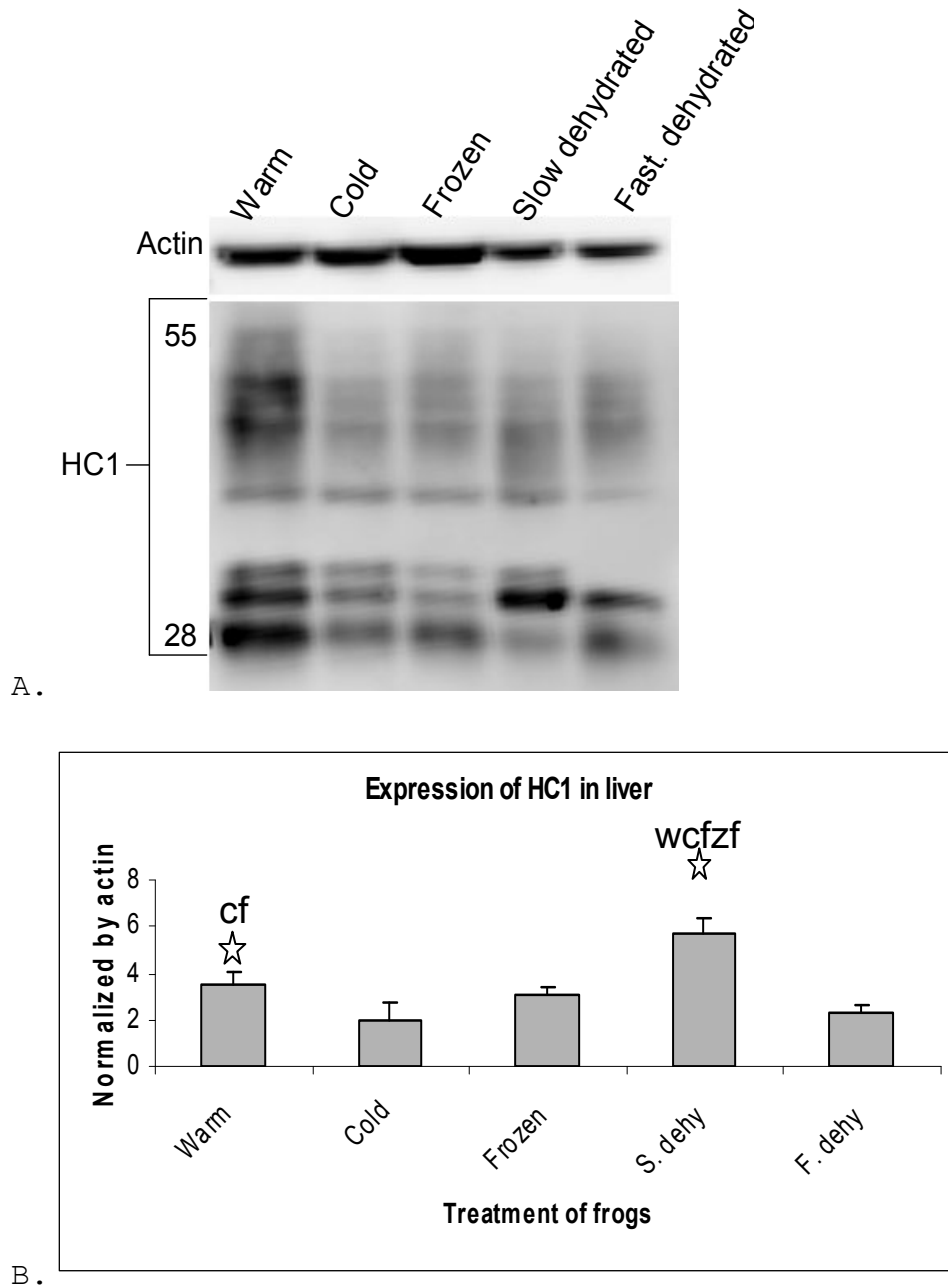


Figure 10. HC-1 expression in liver. A) Triplet bands from 28 kDa all conditions except fast dehydrated tissue. The  $p$ -value is 0.00001 by ANOVA test ( $n=3$ ). The  $p$ -value is  $<0.01$  for cold (c) vs warm (w) and slow dehydrated liver, whereas slow dehydrated has  $<0.01$  with the rest conditions by post hoc test. The  $p$ -value in post hoc test for warm and cold was 0.072. fz and f are frozen and fast dehydrated respectively.

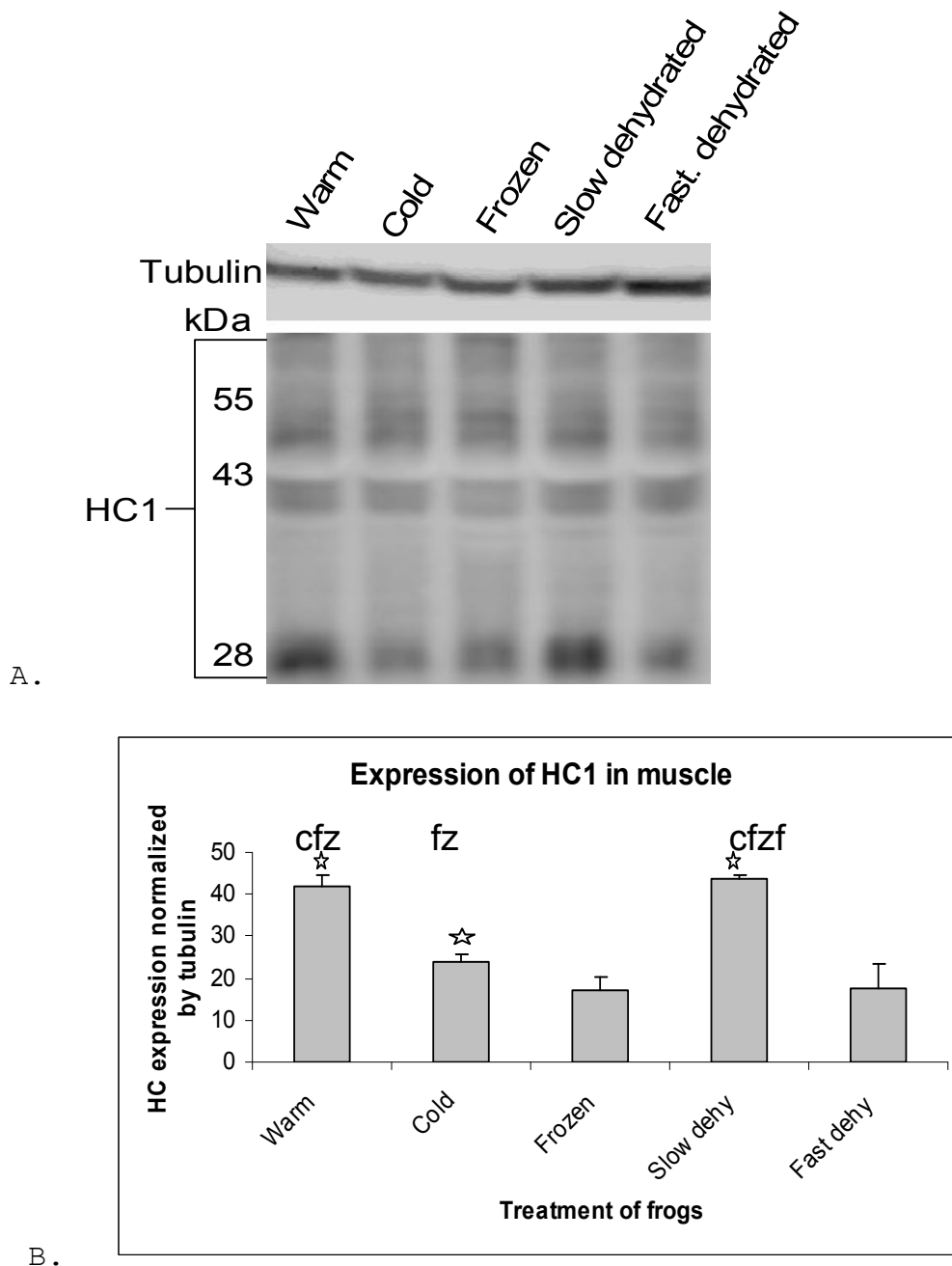
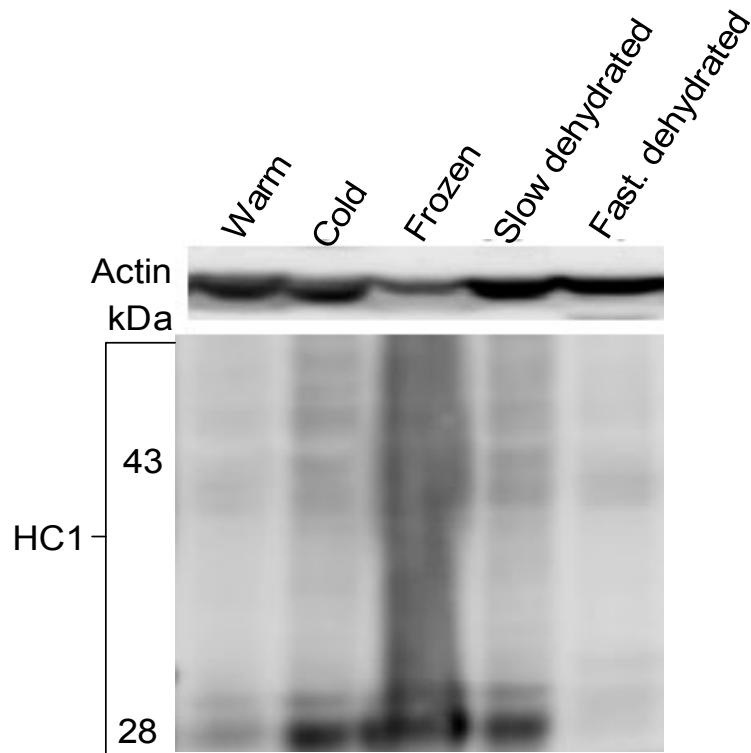
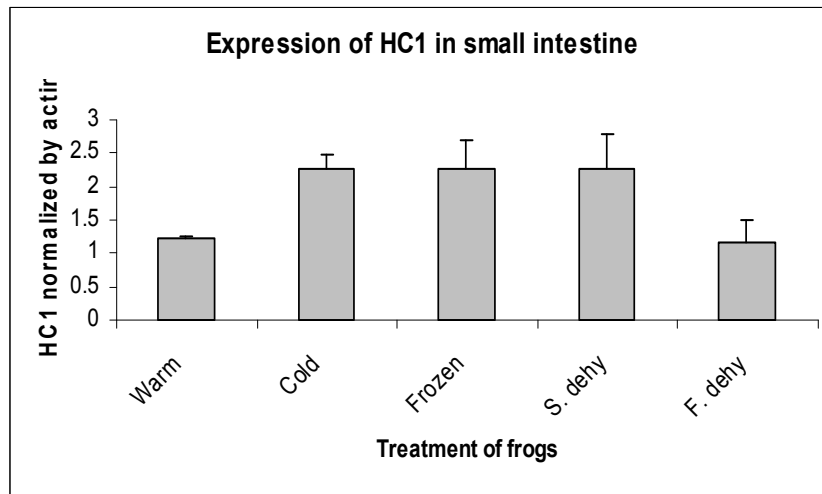


Figure 11. HC-1 expression in skeletal muscle. A) The 30 and 31 kDa bands were absent as they were found in the liver. The higher quantity of nascent proteins of 28 kDa were observed in warm and slow dehydrated tissues ( $n = 3$ ). B) The  $p$ -value was 0.0001 ( $\alpha = 0.05$ ) in ANOVA and post hoc tests among the various treatments of muscles. C, fz, and f means cold, frozen, and fast dehydrated respectively.





A.



B.

Figure 12. HC-1 expression in small intestine. A. Western blot and B. digitized quantity of HC-1 compare to actin in small intestine tissues. The  $p$ -value by ANOVA test is 0.2964 ( $n = 3$ ).

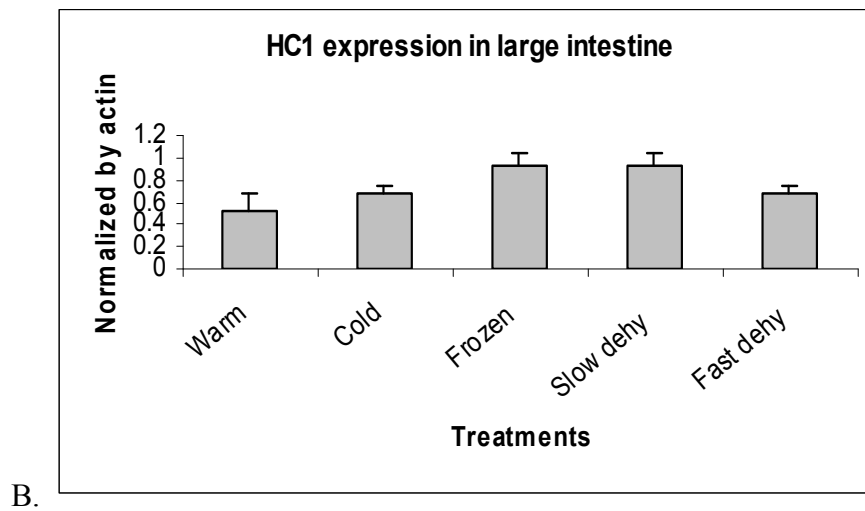
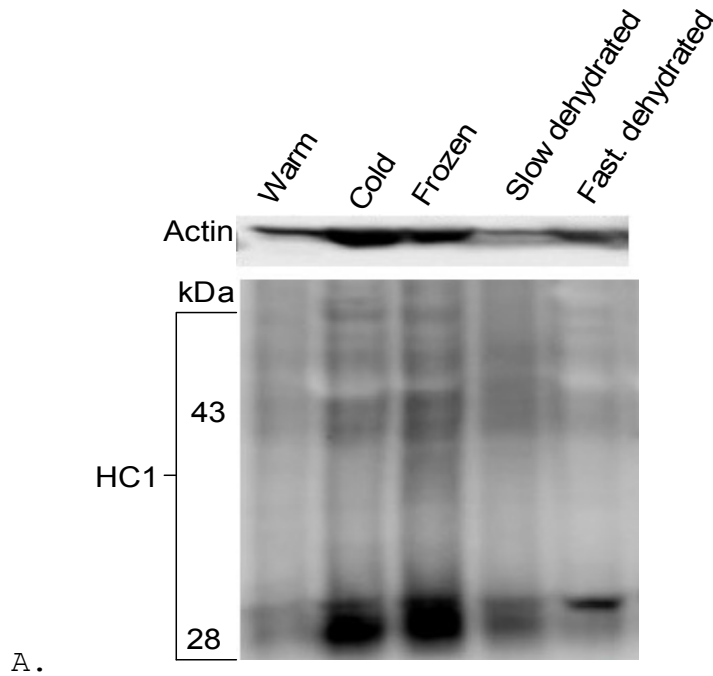


Figure 13. HC-1 expression in the large intestine tissue. A. The 28 kDa band has been shifted to 30 kDa band in the fast dehydrated. B. The  $p$ -value of ANOVA test is 0.1314 ( $n=3$ ).

and fast dehydrated skin. The variation was significant in all conditions with respect to cold skin according to Post Hoc test (fig. 9A, 9B). HC-1 expression in cold was the lowest among all conditions. The remaining conditions had no significant differences for HC-1 expression.

In Western blots for liver, like skin and kidney, the HC-1 protein bands had similar pattern on Western Blot but unlike those, an extra protein band of 32 kDa was found in the liver of warm, cold, frozen, and slow dehydrated animals, but not in the liver of fast dehydrated animals. However, in slow dehydration, the 28 kDa band was very weak and the 30 kDa band was very strong (fig. 10A). A 28 kDa band had low intensity and a 30 kDa band had high intensity in the slow dehydrated animals, whereas the 32 kDa band was missing.

The highest level of HC-1 expression was found in slow dehydrated followed by warm, and lower expression was measured in liver from cold, frozen, and fast dehydrated animals, which were not significantly different from each other (fig. 10A, 10B). The *p*-value for warm vs. frozen was not significant.

The variation in HC-1 expression was significant among different physiological conditions. The expression of HC-1 in skeletal muscle of warm and slow dehydrated frogs was

higher than in cold, frozen, and fast dehydrated frogs (fig. 11A, 11B). The *p*-value for post hoc comparisons was 0.003 for frozen and fast dehydrated with slow dehydrated and warm muscle (fig. 11B).

Skeletal muscle had a different pattern of HC-1 expression in comparison to skin and liver but was similar to kidney. Unlike liver, the 30 kDa and 32 kDa bands were not visible in the skeletal muscle (fig. 11A).

The warm and fast dehydrated small intestine had less intensity of 28 kDa and 30 kDa protein bands and the 32 kDa band was absent like kidney and muscle (fig. 12A). The 30 kDa band had higher intensity than 28 kDa in fast dehydrated large intestine tissue (fig. 12A). HC-1 expression in small intestine appeared to be higher in cold, frozen, and slow dehydrated than the other conditions (fig. 12A); however, the *p*-value by ANOVA test was 0.2964 in small intestine, which was more than 0.05 levels. Similarly in large intestine, there was no significant variation among different physiological conditions (fig. 13A, 13B). The *p*-value for ANOVA test for large intestine was 0.1314, which was more than 0.05.

***Distribution and immunolocalization of HC-1:***

The tissues from five organs--skin, kidney, liver, small and large intestine, and skeletal muscle of warm *H. chrysoscelis*-- were probed with HC-1 antibody to investigate the localization and cellular distribution of HC-1. In skin, HC-1 was found in epidermis, dermis and hypodermis (fig. 14, 16). The region of the parietal layer of Bowman's capsule as well as the thin renal capsule of kidney was labeled with HC-1 antibody in kidney (fig. 14, 15). Liver had some specific signals in septa and sinusoids with HC-1 antibody, as well as some non-specific signals or autofluorescence (fig. 14, 17). In muscle, perimysium, which surrounds the skeletal muscle fibers, expressed HC-1. HC-1 was also present in blood vessels associated with those muscles (fig. 14, 18). HC-1 distribution was similar in both small and large intestines and included labeling of lacteals and/or capillaries in the villous lumen (fig. 14, 19, 20).

In each treatment or physiological condition, the localization of HC-1 expression was consistent; the parietal layer of Bowman's capsule and renal capsule of kidney had high intensity of HC-1 signals. Although intensity of HC-1 expression was not quantified like Western blot, the intensity of expression of HC-1 was

visually lower in slow and fast dehydrated kidney in comparison to cold and frozen kidney (fig. 15).

The whole skin is one of the part of the frog body responsible for evaporation and absorbance of water; the ventral skin is mostly important for water absorption in a tree frog. HC-1 was mostly revealed in gland cells of epidermis, dermis, and hypodermis. The intensity of expression was high in slow and fast dehydrated ventral skin of frog in comparison to warm and cold (fig 16). The differences were also apparent in immunofluorescence images, in which cold and frozen maintains low intensity of signal in comparison to other conditions (fig 16). HC-1 was mostly found in the periphery of mucous glands and serous glands of skin. The ducts of skin glands had vivid signals of HC-1. Hypodermis region was also revealed with HC-1 signals (fig. 14, 16).

Liver is the main organ for gluconeogenesis and glycogenesis, which have direct or indirect roles in glycerol synthesis or sequestration, depending on the environment of a frog. Therefore, it was interesting to investigate whether liver required HC-1 expression in addition of glyceroporins. HC-1 label appeared in the capsular region, septa, and sinusoids (fig.14, 17).

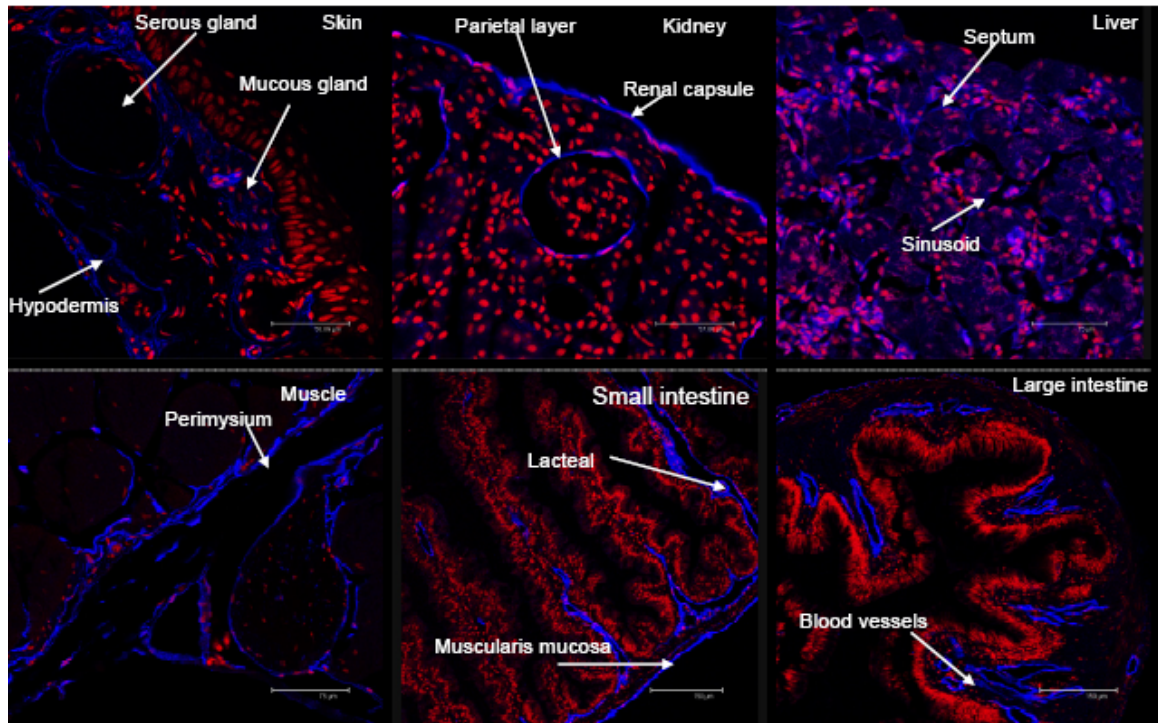


Figure 14. HC-1 localization in warm frog. Immunofluorescence of warm skin, kidney, liver, muscle, small intestine and large intestine with HC-1 antibody and cy5 conjugated secondary antibody. Blue color is HC-1 signal and red (propidium iodide) represents the nuclei (n = 3).

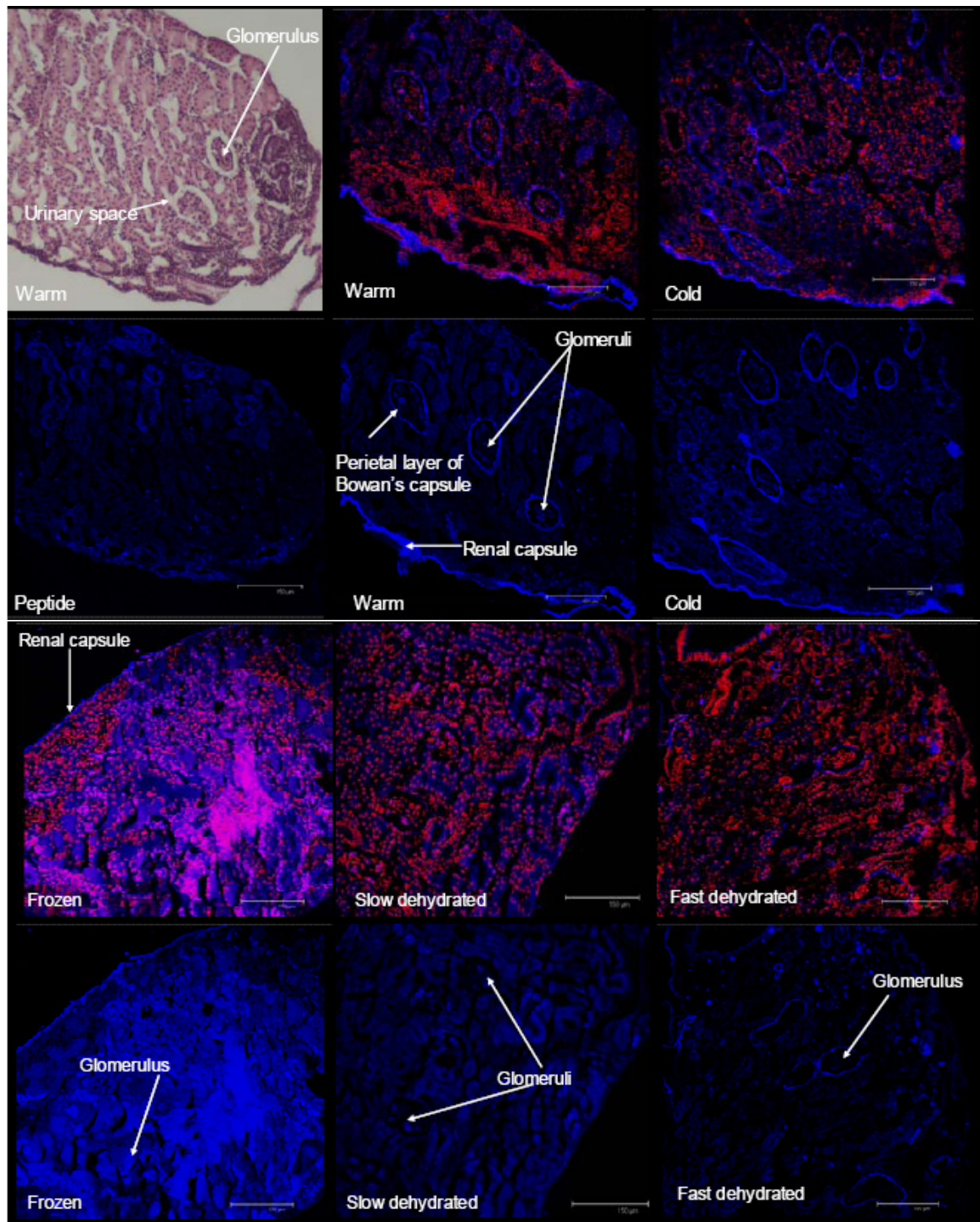


Figure 15. Localization of HC-1 in kidney at different physiological conditions (n = 3). Frozen and slow dehydrated reveal regions of autofluorescence and non specific fluorescence other than glomeruli and renal capsule.



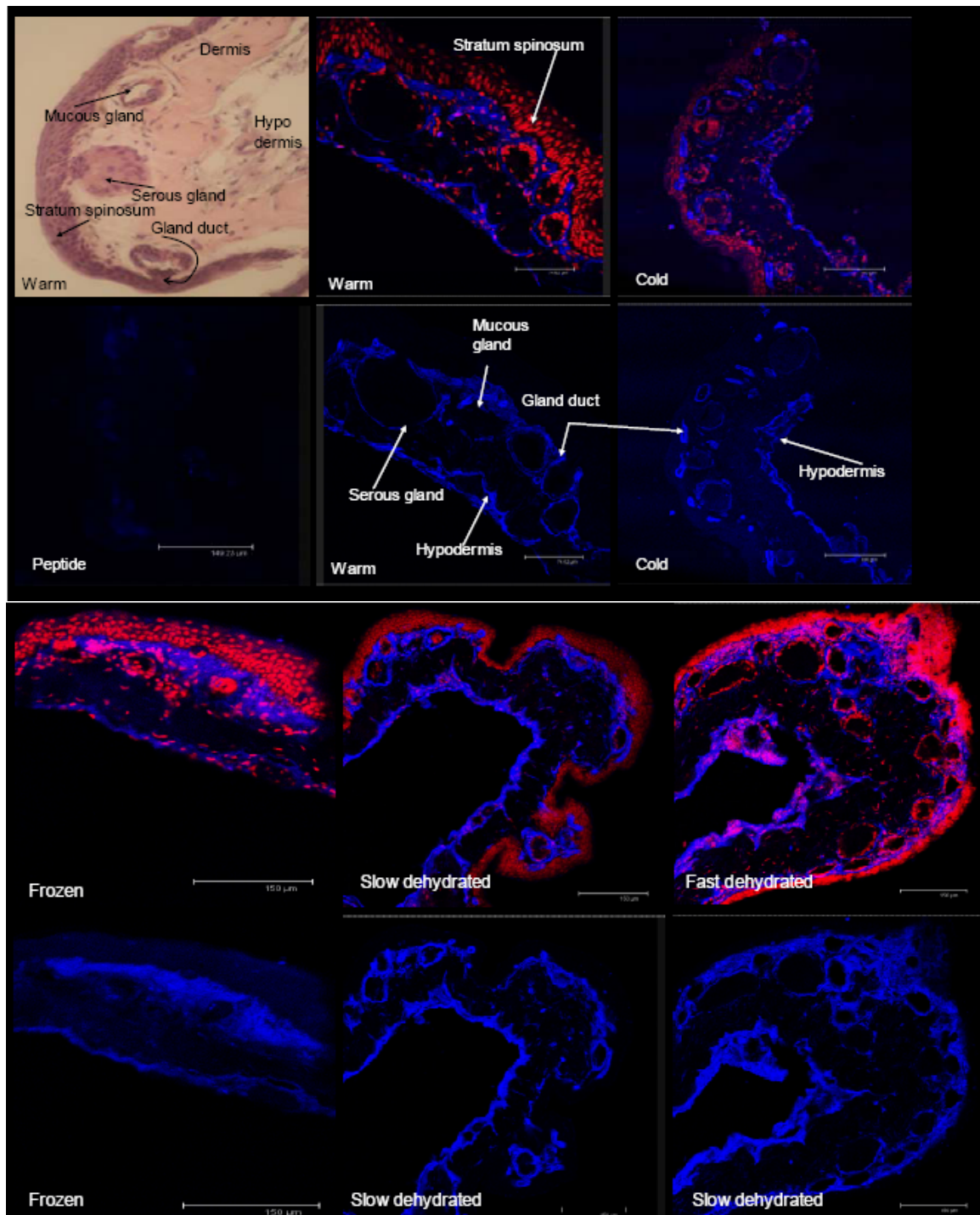


Figure 16. Localization of HC-1 in the ventral skin at different physiological conditions. HC-1 was visualized in the glandular tissues of the ventral skin (n = 3).

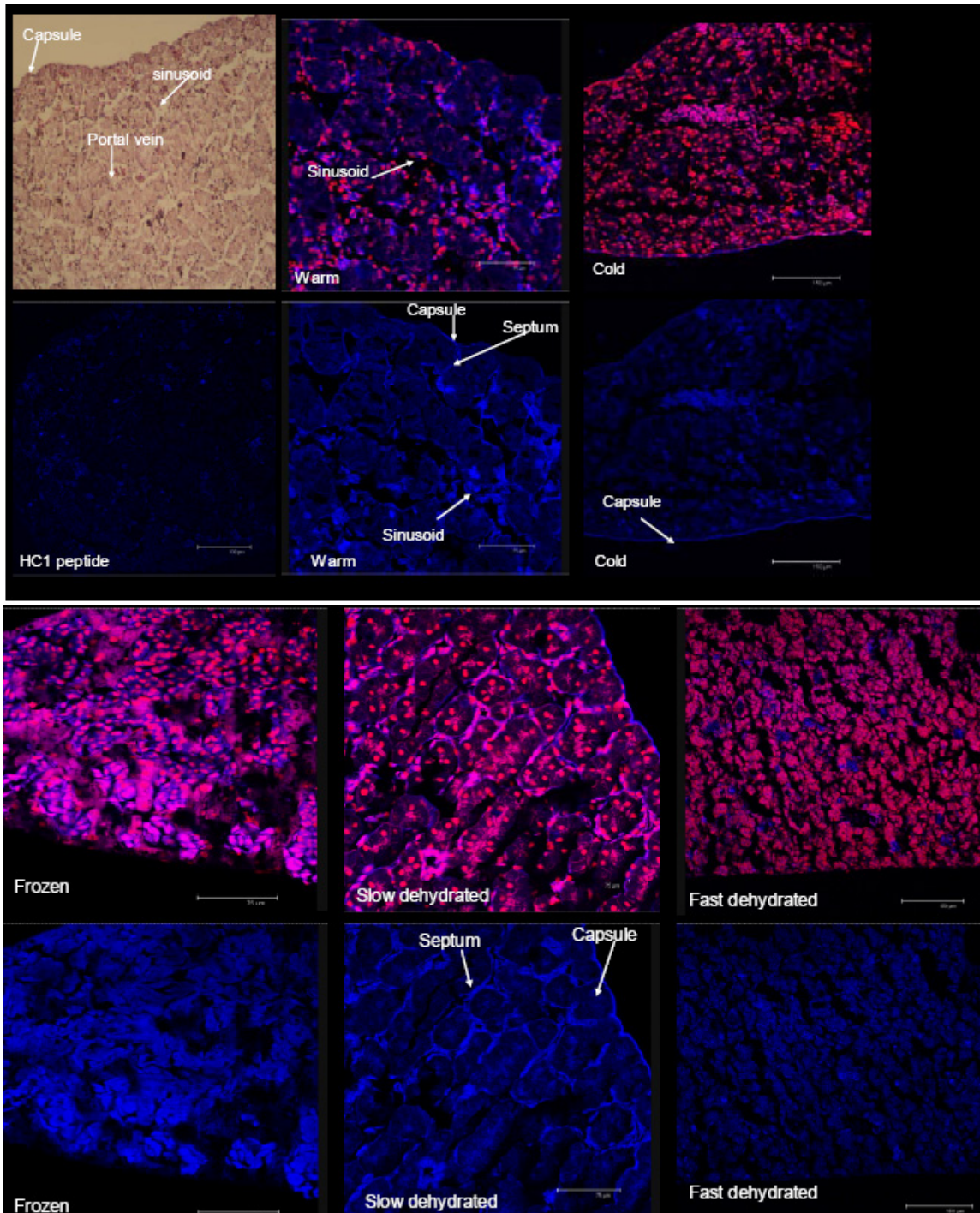


Figure 17. Localization of HC-1 in liver at different physiological conditions. Septum, sinusoids, and capsule had HC-1 expression in liver, but intensity appeared less in cold, frozen, and fast dehydrated frogs (n = 3). Frozen frog liver had autofluorescence showing strong signal, which was confirmed by peptide treated sections.



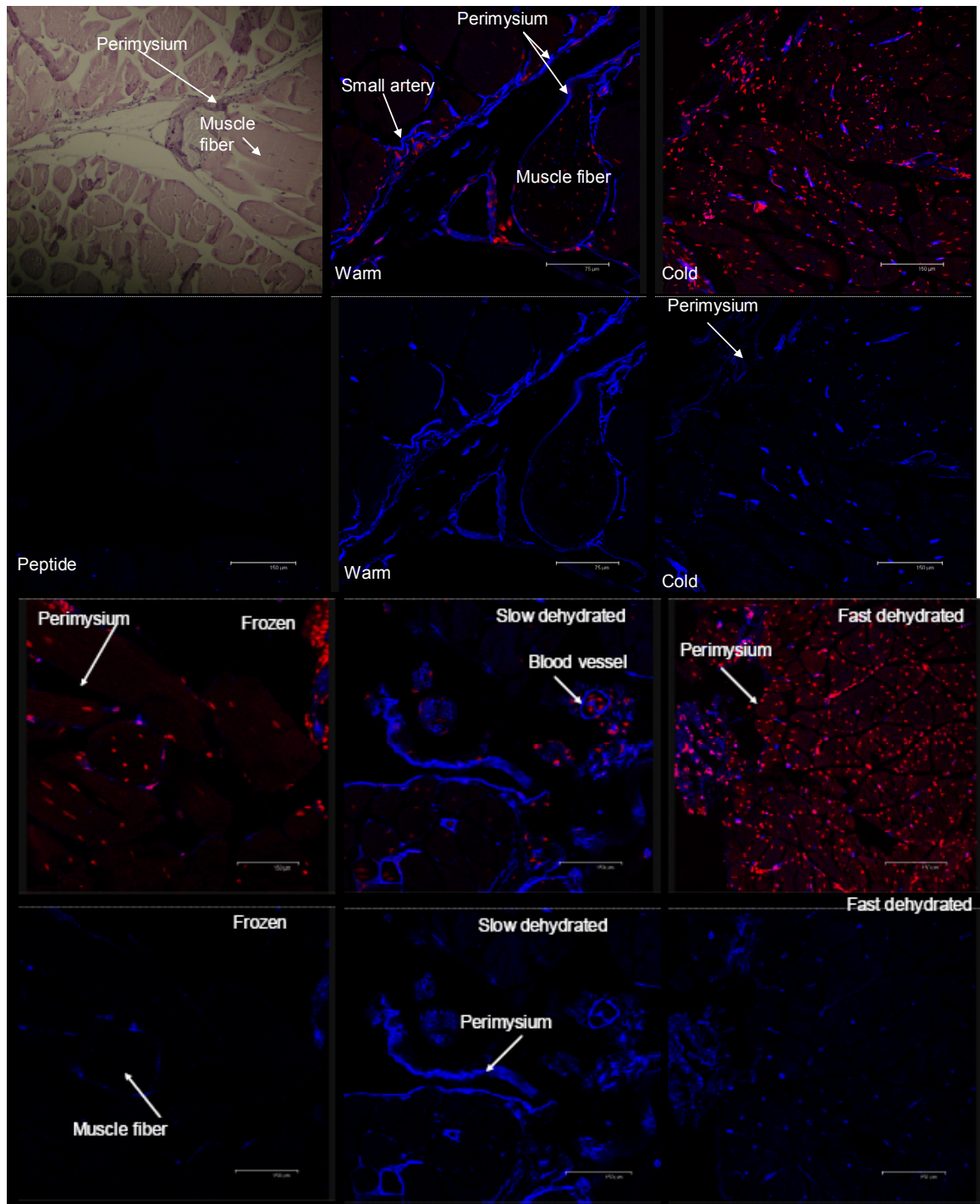


Figure 18. Localization of HC-1 in muscle at different physiological conditions. Blue signal shows the localization of HC-1 and red indicates nuclei (n =3).

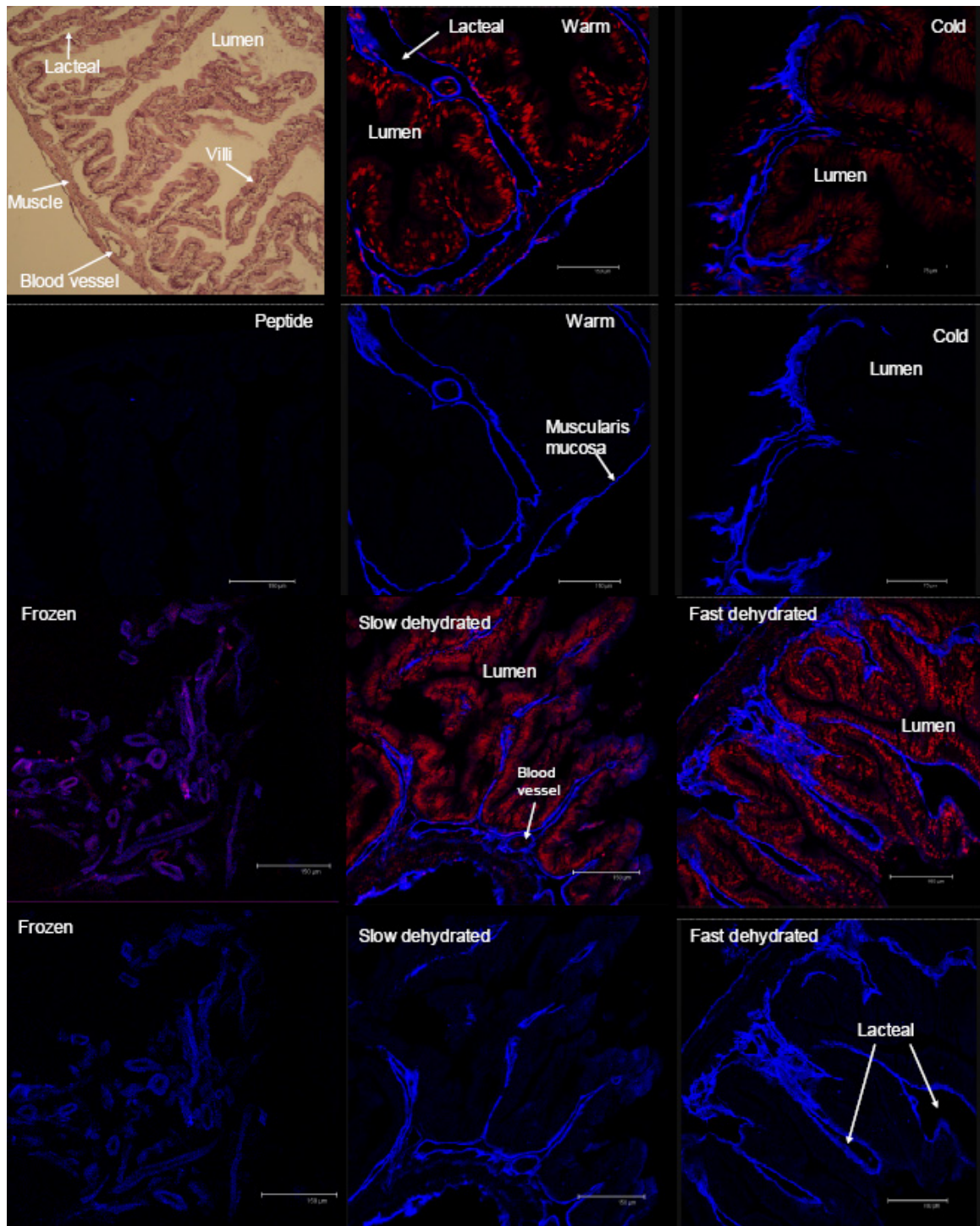


Figure 19. Localization of HC-1 in small intestine at different physiological conditions. Blue color indicates the signal of HC-1, and red indicates nuclei. HC-1 was localized in lacteals, muscularis mucosa, and blood vessels (n = 3).



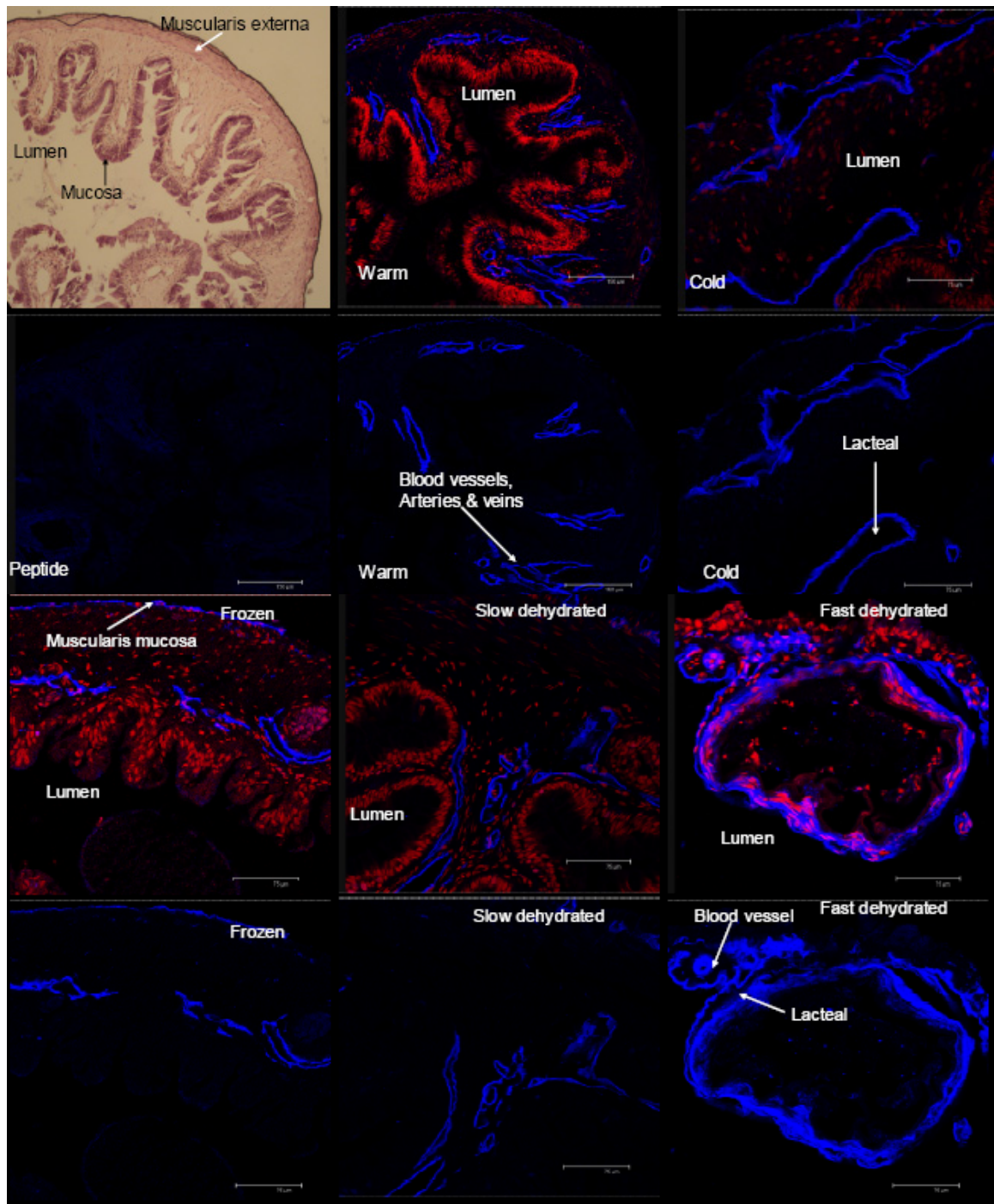


Figure 20. Localization of HC-1 in large intestine at different physiological conditions. Blue color shows HC-1 signal and red (propidium iodide) staining shows nuclei. HC-1 is localized in blood vessels and muscularis mucosa (n = 3).

The intensity of HC-1 protein labeling varied among the five physiological conditions in the liver of the tree frog. The livers from slow dehydrated and warm animals had higher intensity of HC-1 immunoexpression, whereas the fast dehydrated liver had the least expression of HC-1, followed by the tissues from cold animals (fig 17). Localization varied in cold, frozen and fast dehydrated in comparison to warm and slow dehydrated liver. There is no signal in the septum of cold and fast dehydrated liver tissue, whereas signal in the capsule is missing in the frozen liver.

HC-1 immunoexpression in perimysium and blood vessels of muscles were found in all physiological conditions but the intensity of signal varied among these conditions. Warm and slow dehydrated muscle appeared to have the highest intensity of staining with HC-1 antibody in comparison to the others, whereas cold and frozen had weak signals in comparison to fast dehydrated muscle (fig 18).

According to immunofluorescence, HC-1 was expressed in muscularis mucosa, lacteal, and blood vessels of both large intestine and small intestine (fig. 19, 20).

## DISCUSSION

### **Deglycosylated and Glycosylated HC-1 in *H. chrysoscelis***

HC-1 was expressed in ventral skin, kidney, liver, muscle, and intestines (fig.7A) but HC-1 protein bands of varying molecular weights were identified in protein extracts from these those organs. These bands of higher molecular weight represented the glycosylated form required for the maturation of protein post translation (fig 7B). Aquaporin 1 is cotranslationally directed into a four membrane spanning intermediate, which matures into the six membrane spanning topology at a late stage of synthesis (Skach et al. 1994, Lu et al. 2000). Glycosylation of the aquaporin is required for exit from the Golgi body and localization to the cell membrane (Hendriks et al. 2003). Therefore, glycosylation is an important step for generating phyisologically functional aquaporins.

Glycosylation of HC-1 protein was obvious in each organ for each physiological condition of *H. chrysoscelis* (fig.7-13) as N-glycosidase reduced the higher migrating protein bands to lower sizes but the glycosylation pattern

varied in different organs and at different physiological conditions in the current study. According to the Western blots, HC-1 glycosylation was higher in ventral skin, kidney, and liver than in other organs (fig. 7). These data suggest that high glycosylation relates to a higher demand of membrane localization of HC-1, which is required for the homeostasis of water in these organs (fig. 7-10). These organs have important roles in the regulation of body water processing such as evaporation, water exchange, and blood filtration. Blood filtration and tubule water reabsorption in the kidney require high volumes of water transport. Unlike skin and kidney, liver has a vital role in metabolic processes like gluconeogenesis that may increase the osmotic pressure of cells and therefore require water exchange to preserve osmotic balance with the extracellular environment.

Although the glycosylation has been noticed in all organs, the pattern of glycosylation varies among organs and environmental conditions. I suspect that these patterns of glycosylation represent regulatory processes related to expression of functional protein in the membrane. However, it remains to be determined how specific patterns of glycosylation relate to changes in cellular localization or to protein functionality.



### ***Physiological effect on HC-1 expression in kidney***

On the basis of signal intensity of HC-1 protein band and the number of glycosylated bands, HC-1 was significantly expressed in warm, cold and frozen kidney, less in dehydrated kidney, indicating that expression of HC-1 variation depending on the physiological stresses. The cold kidney had more glycosylation of HC-1, which reflects that the cold kidney needs more cell membrane aquaporins in anticipation of freezing and the need to export intracellular water for protection of the cell from ice crystallization (fig. 8A, 8B). I also hypothesize that the higher expression of glycosylated HC-1 in frozen kidney, which is synthesized before freezing, may actually function during recovery after freezing, when the tree frogs require quick transport of water to return the cells to normal condition to perform the cellular functions of the kidney. In terms of slow and fast dehydrated kidney, both nascent and glycosylated HC-1 expression reduced, suggesting that water exchange is low and cells do not need much HC-1 during this time of reduced kidney function (Zimmerman *et al.* 2007).

The specific localization of HC-1 within the kidneys of *H. chrysoscelis* is different from what has been

described for other vertebrates. HC-1 was most strongly expressed in the region of parietal layer of Bowman's capsule, which surrounds the urinary (capsular) space (fig. 14). The Bowman's capsule is not directly involved in renal filtration, but collects filtrate as it forms (fig. 21). It is thus not clear why this structure should require a high exchange of water. However, Kamiie *et al.* (2002) have reported aquaporin 1 mRNA in human glomeruli but not in rat glomeruli. They revealed 28 kDa and 35 kDa bands corresponding to unglycosylated and glycosylated aquaporin 1 proteins in human glomeruli. Immunoreactive aquaporin 1 was demonstrated almost exclusively in the mesangium of the human glomeruli by immunohistochemistry. The endothelium of glomerular capillaries was only partly immunostained while podocytes and Bowman's capsule epithelia were not immunolabeled (Kamiie *et al.* 2002).

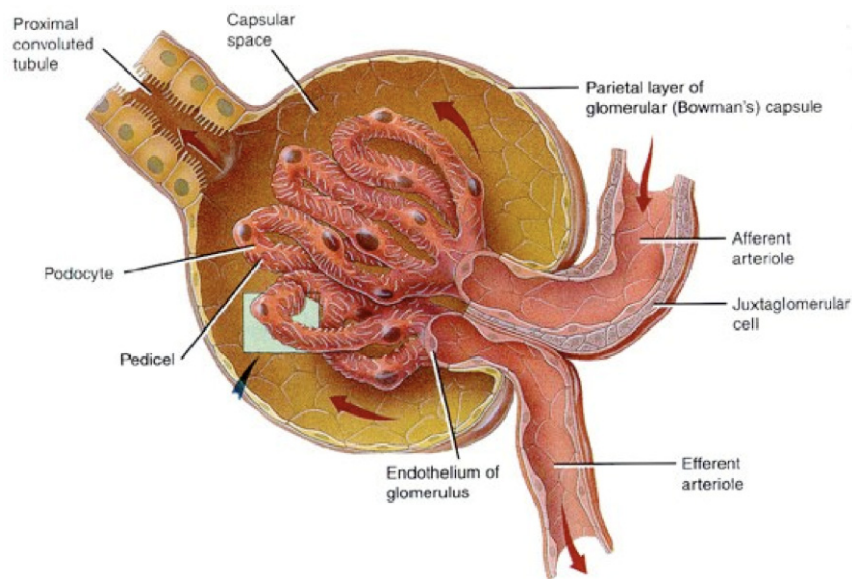


Figure 21. A diagrammatic presentation of Bowman's capsule of kidney (source: [www.marlerblog.com](http://www.marlerblog.com))

Perhaps, the cells need HC-1 to accommodate their own water exchange, as they come in contact with the protein-free fluid that is filtered through the glomerular capillaries. HC-1 was also expressed in the renal capsule, the cover of kidney surface made of connective tissues and collagen fiber. In mammals, the capsule is a tough outer coating to the kidney that balances the volume and pressure of kidney. In amphibians, however, the renal capsule is much thinner and does not likely have such a regulatory function.

Unlike *H. chrysoscelis*, in mammals, aquaporin 1 is expressed in the apical and basolateral membranes of the epithelial cells of the loop of Henle, especially in the initial descending limb, where it serves in the reabsorption of water (Schnermann *et al.* 1998). However, there is no loop of Henle in frogs.

#### ***HC-1 expression and localization in the ventral skin***

Expression of HC-1 was assessed in reference to endogenous actin expression in the frog skin. Significant variation was found in the expression of HC-1 among different physiological conditions (fig 9B). Unlike the kidney, HC-1 synthesis was reduced in the cold frog skin, which might be either decreased transcription or

translation of HC-1 in cold skin in comparison to slow and fast dehydrated skins. The double band of HC-1 protein in the Western blot, just above the 28 kDa band, occurred for skin from all physiological conditions except frozen animals. However, it appears that the nascent protein, 28 kDa, is converted to a 31 kDa band either after phosphorylation or glycosylation in cold tissues without further synthesis of 28 kDa proteins, as it was found in less intensity in cold and frozen skins (fig 9). It might be associated with the reduction of HC-1 on membrane to suppress the further water transport or expression of glyceroporins to reduce the high exchange of water, which is performed by HC-1. The HC-1 function might have been substituted by HC3, a glyceroporin, which has also less water transport and more glycerol exchange ability (Zimmerman et al. 2007). The 31 kDa band of frozen skin might have disappeared due to no demand of water transport during freezing environment. The expression of glycosylated HC-1 proteins of slow and fast dehydrated skin is opposite to the level of HC-1 expression pattern in cold and frozen skin; slow and fast dehydrated ventral skin had high levels of glycosylation, especially at lower molecular weights. In the case of slow dehydrated animals, we suggest that a high demand of HC-1 in ventral skin relates to the

possibility of water uptake across this organ if water becomes available. Therefore, high level of a 28 kDa band and intensive glycosylation of HC-1 is observed in the slow dehydrated skin. In the fast dehydrated skin, a 31 kDa glycosylated band is much less prominent; we speculate that this may reflect the time course of slow vs. fast dehydration, but we cannot attribute specific functionality to specific patterns of glycosylation (fig. 9A).

Immunofluorescence data indicate that HC-1 was expressed mostly in dermis and hypodermis of the skin, with the exception in the epidermis where gland ducts are located. Dermis and hypodermis are rich in gland cells with spongy and fat body layers where water content is very high. The serous and mucous glands in dermis region of skin are rich in HC-1 expression (fig. 14, 16). Serous secretions have a low viscosity, i.e. they are rather "watery". Mucous secretions have a high viscosity, i.e. they are rather "slimy". The secretory region of serous gland is well-stained with HC-1 antibody. Likewise, the contents of the secretory vesicles in the cells forming mucous gland are also strongly labeled. These empty-looking vesicles give the apical cytoplasm of mucus-producing cells a distinct "foamy" or "frothy" appearance.

The hypodermis is rich in adipose tissue, which contains little water. However, the HC-1 is prominently visible in hypodermis of ventral skin in all physiological conditions (fig. 14, 16). Therefore, the current data indicate a high demand of HC-1 to maintain the water requirement of secretory glandular tissues in different regions of skin. This pattern of expression of an aquaporin from the aquaporin 1 family has not been reported before. Suzuki *et al.* (2007) have reported aquaporin 1 in the ventral skin of *H. japonica* without detailed information about its localization.

#### ***HC-1 expression and localization in liver***

Significant variation was found among the physiological conditions in expression of HC-1 in liver. HC-1 expression was significantly increased in slow dehydrated liver, signifying its biological importance to maintain homeostatic water exchange with increasing concentration of glucose (fig.10B). Like skin, HC-1 increased in slow dehydrated and reduced in cold liver significantly. Liver expresses other aquaporins in addition to HC-1, including glycerol transporting glyceroporins, and these may function especially during the cold, when liver is active in glycerol synthesis (Zimmerman *et al.* 2007).

Other aquaporins, such as HC3, which is also expressed in liver (Zimmerman *et al.* 2007), may facilitate glycerol (cryopreservative) movement into cells where it is used, or out of cells (hepatocytes) where it is generated.

Western blots of HC-1 from liver were unique. Triplet bands from 28 kDa were obvious in Western blots from all conditions except fast dehydrated liver. The expression of 30 kDa glycosylated protein was highest in warm and slow dehydrated conditions, suggesting a high demand for HC-1 in the membrane in these conditions (Hendriks *et al.* 2004, review Takata *et al.* 2008) (fig 10A). The disappearance of the largest of the three bands (approx 32 kDa) in the fast dehydrated state presumably represents a suppression of some component of glycosylation, and this was specific to liver only; we speculate that this could protect the liver from over-dehydration during conditions of rapid desiccation (fig. 10A).

HC-1 was expressed in liver capsule and septum, though the signal in capsule was weak in fast dehydrated liver (fig. 14, 17). The septa are made of the connective tissue of the capsule, which separates the lobules of the liver. The sinusoids, which are irregularly dilated capillaries composed of a discontinuous layer of fenestrated endothelial cells, also had HC-1 expression. I speculate



that HC-1 functions along with other aquaporins in the liver. As noted above, liver is an important organ for glycerol synthesis and distribution during cold conditions, and for that function the liver needs a aquaglyceroporin (like HC3) rather than HC-1. The presence of aquaporin 1 in human liver sinusoid and endothelium tissue of blood vessels (Talbot *et al.* 2003, Mobasheri and Marples, 2004, Portincasa *et al.* 2008) is consistent with the presence of aquaporin 1-like HC-1 in the *H. chrysoscelis*.

#### ***Physiological effect on HC-1 expression in muscle***

In muscle, we did not observe the strong signals for 30 and 31 kDa bands as we observed in liver. However, we did observe varying expression of the nascent 28 kDa protein, with higher intensity of staining in warm and slow dehydrated muscles (fig. 11A). The physiology of muscle is different from other organs. The high synthesis of HC-1 was observed in warm and slow dehydrated frog muscle. It might be on the basis of need of water exchange. The pattern of HC-1 glycosylation in muscle was not similar to other tissues for example 31 and 32 kDa bands were missing. Similar variability has been described in other animals, for example, the pattern of aquaporin 1 expression and

glycosylation differs among reproductive tissue of mice (Lu *et al.* 2008).

HC-1 expression was detected in perimysium of muscle fiber bundle. Perimysium is a sheath of connective tissue which groups individual muscle fibers into bundles or fascicles. Outside of the fibers themselves, HC-1 expression was observed in small arteries (fig. 14). The metabolism in muscle may vary in cold, frozen, and fast dehydrated frog's skeletal muscles since animals are at the rest position with minimal activities. As a result water exchange activities are reduced to a basal level. Therefore, the data indicate that the HC-1 expression might be less at cooling or lost after fast dehydration in the muscle of *H. chrysoscelis*.

#### ***Regulation of HC-1 in small and large intestine***

The 28 kDa and 30 kDa bands including other glycosylated bands have light signals in warm and fast dehydrated small intestine in comparison to cold, frozen, and slow dehydrated small intestine, indicating reduced exchange of water in these conditions. Although the cold and frozen small intestine appeared to have more quantity of HC-1 in immunofluorescence, that variation was not significant in Western blots among the different

physiological conditions. This might reflect a continuing demand for water exchange, for digestion, to protect the intestine from freezing, and to revive the intestinal cells during freeze recovery when extracellular ice melts.

The blood vessels/lacteals of the intestines were strongly labeled with HC-1 antibody (fig. 19, 20), consistent with the pattern of AQP1 expression in human and rat small intestine (Nielson et al, 1993, review Tonghui and Verkman 1999). The expression of HC-1 in the small and large intestine was similar to previously published papers. Aquaporin 1 was present in endothelial cells of capillaries and small vessels in the digestive system (Nielson et al. 1993, Kayoma et al. 1999, Hurley et al. 2001, Ma et al. 2001, Matsuzaki et al. 2004).

I could not definitely say whether HC-1 expression occurred in blood vessels or in lymphatics. In the blood vessels, HC-1 could facilitate trans-endothelial transport of water. A rather controversial role for aquaporin 1 in the transport of chylomicrons, lipoproteins that are transported via the lacteals during intestinal digestion, has been speculated on (Matsuzaki et al. 2004, Tso et al. 1985). There was little difference in the HC-1 expression in small and large intestine (fig. 18, 19).

### **Comparison of HC-1 expression among organs and species**

In several species of mammals, such as rat, mice, human, horse, and dogs, aquaporin 1 is widely expressed across the different organs, but is usually considered a “housekeeping” aquaporin. Aquaporin 1 is constitutively expressed and not highly regulated like aquaporin 2 and 3. I found that the widespread distribution of expression was also the case in *H. chrysoscelis* because aquaporin 1- like HC-1 was present in all organs and tissues examined. For many of the organs, the specific localization of HC-1 is also similar as for mammalian aquaporin 1. For example, aquaporin 1 was reported in the endothelial cells of lacteals (Nielsen *et al.* 1993), capillaries, and small vessels of small and large intestine in human (Matsuzaki *et al.* 2004), and aquaporin 1 was abundant in the endothelial cells of capillaries and small vessels in human liver (Matsuzaki *et al.* 2004), similar to HC-1. Aquaporin-1 (AQP1) was widely distributed in endothelial cells of capillaries and small vessels as well as in the central lacteals in the small intestine, pancreas, liver, and bile duct (Matsuzaki *et al.* 2004). However, in other cases the localization of expression in the tree frog is different from mammals. For example, aquaporin 1 has been reported in epithelium of intrahepatic bile duct of liver (Nielsen

et al. 1993), and in the colon Crypt epithelium of human (Hasegawa et al. 1994) but HC-1 was not found in these locations of *H. chrysoscelis*. An interesting question from the current study is the role of HC-1 in connective tissues. For example, this aquaporin was expressed in renal and hepatic capsules, both of which are thin connective tissue sheets. HC-1 may be involved in cellular homeostasis in these organs.

In addition to some differences in localization, this study also suggests that amphibian aquaporin 1-like HC-1 is regulated according to physiological demand to a greater extent than in mammals. That regulation involves both overall changes in expression and patterns of glycosylation depending on the mode of water exchange as for example liver, skin, and kidney. In the animals we studied, a newly translated 28 kDa protein of HC-1 was found in all organs and treatments. In contrast, specific glycosylated forms of HC-1, e.g., the bands at 30 kDa and 32 kDa, were present only in particular organs or circumstances. The glycosylation pattern and number of glycosylated bands varied among examined organs of *H. chrysoscelis*; this has also been reported for aquaporin 1 in kidney, testis, epididymis, vas deferens, ventral prostate, and seminal vesicles of the mouse (Lu et al. 2008).

HC-1 was regulated by environmental and physiological conditions of *H. chrysoscelis*. That regulation appears to be organ specific, suggesting that regulation of HC-1 confers specific water permeability properties to different organs. HC-1 appears to be expressed in many tissues where constitutive high demand of water exchange is required. This situation is reminiscent of findings for microorganism, which expresses aquaporins for water exchange primarily in species likely to need high demand for water exchange during conditions like freezing (Tanghe *et al.* 2006).

We were interested in whether patterns of total HC-1 expression indicated a similarity of regulation in cold conditions (cold or frozen animals) and in dehydration (slow and fast dehydrated animals), as freeze tolerance has been postulated to have evolved from more general mechanisms of response to dehydration. We did not detect consistency between these two circumstances in the expression of HC-1 among these tissues. For example, although total HC-1 expression was either low or high in skin, liver, and muscle from both frozen and fast dehydrated animals, expression patterns differed in many other comparisons between slow dehydrated and cold animals, or fast dehydrated and frozen animals. These differences

might be due to seasonal shifts in physiology or to the durations of the different treatments. Alternatively, it is possible that the response to dehydration occurs by different mechanisms, possibly involving different regulatory signals, than the response to cold. For example, although extracellular osmolality might increase in both cold and dehydration, the solutes accounting for that change differ (glycerol vs. ions), and in one case (dehydration) body fluid volume is diminished, whereas this is not the case in the cold.

#### **Future research**

Although this research provides a descriptive view of aquaporin 1 expression in *H. chrysoscelis*, that descriptive view opens the doors to further works to determine the physiological role of HC-1 in *H. chrysoscelis* and other animals. More detailed knowledge about HC-1 localization-- both the specific cells where it is expressed and the location within the cells-- will better reveal the function of aquaporin 1 in various organs. Our research suggests that HC-1 may work in coordination with other aquaporins to regulate the cellular and tissue demand for water exchange. A comparative study of HC-1 with the other aquaporins such as HC-2 and HC-3 will further elucidate the physiological

basis for fluctuations in expression of HC-1 in *H. chrysoscelis*. Additionally, future research is required as to how specific patterns of glycosylation relate to changes in cellular localization and protein functionality. Which glycosylated bands are localized in the plasma membrane? Are other means of protein processing also important, such as phosphorylation and ubiquitination? Such studies might relate to the question of whether HC-1 is newly synthesized, or perhaps recycled, during pre and post-environmental stress; what processes or transcription, translation, and post-translational processing result in changing expression? A cell culture system of *H. chrysoscelis*—perhaps of cells isolated from skin—would be a good *in vitro* research to explore mechanisms of regulation of HC-1 expression.

### **Limitations of findings**

In the current research, there were some limitations in our ability to produce and interpret the data. Due to limited physiological and anatomical literature about our study species, it was difficult even to speculate about the role of HC-1 in a number of organs. The limited resolving power of light microscopy prevented us from identifying specific localization of signals within cells, such as the



presence in membrane vs. cytoplasm. As amphibian histology is different from the mammalian, identification of cells and tissue on H&E stained organs was not always unambiguous. In the lack of phosphorylated, glycosylated, ubiquitinated HC-1 antibody, the different bands of HC-1 proteins were not addressed. HC-1 may play a role in transport of ions (through the central pore) in addition to water, and were not able to consider this possibility, which is not confirmed for HC-1.

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